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PHYTIN AND PHOSPHORIC ACID ESTERS OF INOSITE

R. J. ANDERSON

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TECHNICAL BULLETIN No. 19.

PHYTIN AND PHOSPHORIC ACID ESTERS OF INOSITE.

R. J. ANDERSON.

SUMMARY.

1. The barium salt of phytic acid, $C_6H_{24}O_{27}P_6$, obtained from very dilute hydrochloric acid or 10 per ct. phytic-acid solutions corresponds to the general formula $C_6H_{18}O_{27}P_6M_3$. Apparently the same relation holds for other binary cases.
2. From neutral or alkaline solutions salts of the general formula $C_6H_{12}O_{27}P_6M_6$ are obtained.
3. From very dilute acetic-acid solutions intermediate salts are formed.
4. The acids isolated from the organic-phosphorus compound known as phytin, derived from different sources, have been shown to be identical. The constitution of the substance, however, still remains in doubt.
5. Attempts to synthesize phytic acid by acting on inosite with dry phosphoric-acid in vacuum at 140°–160° C. failed. In this reaction the tetra-phosphoric acid ester of inosite was produced.
6. The tetra-phosphoric ester was found to be very similar to phytic acid. Its barium salt obtained from very dilute hydrochloric acid solutions corresponds to the general formula $C_6H_6(OH)_2O_4[(PO_3H)_2M]_2$.

PREVIOUS INVESTIGATIONS.

In continuation of the physiological investigation concerning the metabolism of the organic-phosphorus compound known as phytin, which has been and is being carried out at this institution by Dr. Jordan, a closer study of the chemical properties of this substance, phytin, became necessary. Much work has already been done and reported on this subject by various investigators. Definite information, however, concerning different kinds of salts formed by the free phytic acid or inosite phosphoric acid is seldom met with in the literature. Frequently impure salts have been analyzed.

Posternak, who first successfully prepared phytin in pure form,[1] also studied its chemical properties. Among the salts mentioned[2] is one, calcium-magnesium, as well as one crystalline, calcium-sodium, double salt, for which he gives the formula $2C_2H_4P_2O_9Na_4 + C_2H_4P_2Ca_2 + 8H_2O$. Winterstein[3] describes a calcium-magnesium compound which, after removing the calcium with oxalic acid and precipitating with alcohol, contained 42.24 per ct. P_2O_5 and 12.97 per ct. MgO. Patten and Hart,[4] working in this laboratory, isolated from wheat bran an impure magnesium-calcium-potassium com-

[1] *Rev. gen. Bot.* 12: 5; *Compt. Rend.* 137: 202.
[2] *Compt. Rend.* 137: 337 and 439.
[3] *Ber. deut. chem. Ges.* 30: 2299.
[4] *Amer. Chem. Journal* 31: 566.

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pound. Levene[1] describes a semi-crystalline barium salt which corresponds to a tetra-barium phytate. Vorbrodt[2] mentions a crystalline barium salt obtained by partially neutralizing phytic acid with barium hydroxide and evaporating in vacuum, to which he assigns the formula $C_{12}H_{26}O_{46}Ba_7P_{11}$. Although crystalline, this compound was undoubtedly impure. By neutralizing the mother-liquor from the above with barium hydroxide he obtained an amorphous precipitate of the composition C 5.75 per ct., H 0.77 per ct., Ba 52.97 per ct., P 11.60 per ct. This corresponds approximately with a hexa-barium phytate.

Of the several salts mentioned in this paper some were obtained from commercial phytin and from an organic-phosphorus magnesium compound by precipitating with barium chloride and barium hydroxide; others were prepared from previously purified phytic acid. These products will be more fully described in the experimental part.

The tri-barium phytate, $C_6H_{12}O_9\ [(PO_3H)_2Ba]_3$, is obtained pure as an amorphous white powder by repeatedly precipitating barium phytate in 0.5 per ct. hydrochloric acid with a like volume of alcohol. It may also be obtained in crystalline form by dissolving the amorphous salt in a 10 per ct. solution of phytic acid in which it is very soluble and from which it again slowly crystallizes out on standing at ordinary temperature.

A penta-barium phytate, $C_6H_{14}O_{27}P_6Ba_5$, is obtained when a solution of the tri-barium phytate in 0.5 per ct. hydrochloric acid is neutralized with barium hydroxide and then made faintly acid with acetic acid.

The penta-barium ammonium phytate, $C_6H_{12}O_{27}P_6Ba_5(NH_4)_2$, is obtained when the above mentioned amorphous tri-barium salt is digested with dilute ammonia.

The penta-magnesium ammonium phytate, $C_6H_{12}O_{27}P_6Mg_5(NH_4)_2$, is thrown down as a white amorphous precipitate when excess of magnesia mixture is added to an aqueous solution of phytic acid, or when ammonium phytate is precipitated with magnesia mixture.

A tetra-cupric di-calcium phytate, $C_6H_{12}O_{27}P_6Cu_4Ca_2$, in nearly pure form is obtained when a slightly acid solution of calcium ammonium phytate is precipitated with excess of copper acetate. If the magnesium ammonium phytate is precipitated under the same conditions an impure compound is obtained which contains about 1 per ct. Mg, 0.6 per ct. N, 34 per ct. Cu and 15.6 per ct. P. No effort was made to obtain these salts pure. It was only desired to find out to what extent other bases were removed when precipitating with copper acetate.

Starkenstein[3] claims that commercial phytin always contains free inosite together with inorganic phosphates and that merely drying the substance at 100° C. causes nearly complete decomposition into inorganic phosphate and free inosite.

That phytin is so easily decomposed seemed very improbable, as several months' work on the substance has shown that it is relatively

[1] *Biochem. Ztschr.* 16: 309.
[2] *Anzeiger Akad. Wiss. Krakau* 1910, Series A: 414.
[3] *Biochem. Ztschr.* 30: 59.

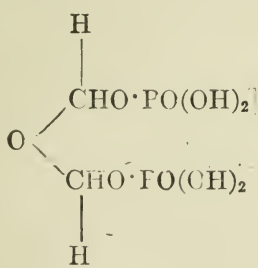
stable when pure and when no mineral acids are present. Moreover, Contardi[1] reports that when phytin is heated in an autoclave with pure water for several hours to a temperature of 200° C. only very small quantities of inosite could be isolated.

In order to determine if inosite is present in determinable quantity 100 gm. of commercial phytin in the form of the acid calcium salt, which had been imported from Europe and kept in the laboratory for several years, was shaken up with 1 liter of water, filtered at once and washed with water. The filtrate was precipitated with barium hydroxide, again filtered and the excess of barium precipitated with carbon dioxide, and the filtrate from the latter evaporated on the water-bath. In the very slight residue which remained, consisting mostly of barium carbonate with a trace of barium chloride, no trace of inosite could be detected by the most painstaking method of isolation. Of the same phytin 100 gm. was dried to constant weight at 115° C. and was then treated in the same manner. Even here no trace of inosite could be obtained. Subjecting to the same treatment 50 gm. of the same phytin, after previously mixing with 0.5 gm. inosite, resulted in the recovery of 0.4 gm. inosite.

This proves that phytin is by no means so easily split as Starkenstein claims. The results in his case may have been due to other causes besides mere drying at 100° C.

The same author (loc. cit.) also states that when phytic acid is precipitated with ammoniacal magnesia mixture it is not the magnesium ammonium compound which is formed, but only the difficultly soluble magnesium phytate. This is an error. Under these conditions the previously mentioned penta-magnesium ammonium phytate, $C_6H_{12}O_{27}P_6Mg_5(NH_4)_2$, is formed.

For the free phytic acid Posternak[2] proposed the empirical formula $C_2H_3O_9P_2$ which he considered to have the following constitution:

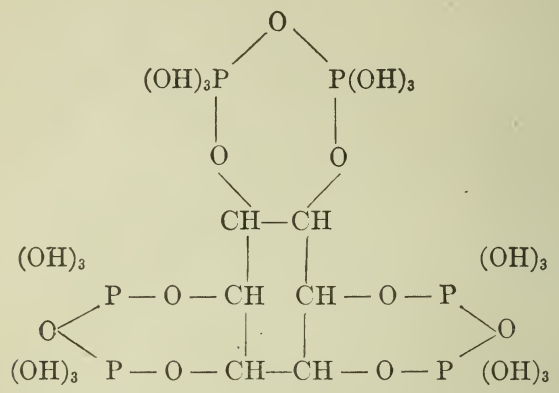


which finds expression in the name "anhydro-oxymethylen diphosphoric acid."

As is well known the free acid, as well as its salts, is easily split under the influence of dilute mineral acids into inosite and orthophosphoric acid. This fact and the discovery by Neuberg[3] that

[1] *Atti R. Accad. dei. Lincei*, Roma (5), 18, I: 64.
[2] *Compt. Rend.* 137: 439.
[3] *Biochem. Ztschr.* 9: 551 and 557.

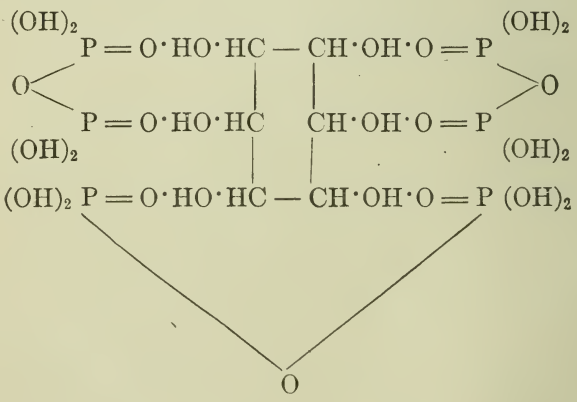
both inosite and phytin yield furfurol when distilled with phosphorus pentoxide and phosphoric acid, respectively, lead him to believe that the inosite ring exists already formed in phytin. In accordance with this view he proposed the following structural formula for the acid:



This is just treble the molecular weight of the anhydro oxymethylen di-phosphoric acid of Posternak.

Suzuki and Yoshimura[1] considered that phytic acid was the hexa-phosphoric acid ester of inosite.

Starkenstein[2] believes that phytin represents a complex pyro-phosphoric acid compound with inosite and he proposes the follcw-ing constitutional formula:



Vorbrodt (loc. cit.) proposes still another formula.

[1] *Bull. College of Agric.*, Tokyo, 7: 495.
[2] *Biochem. Ztschr.* 30: 56.

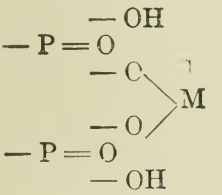
It is impossible at the present time to decide definitely between any of the above constitutional formulas, as the substance has not yet been synthesized in the laboratory.

As represented by the empirical formula, $C_6H_{24}O_{27}P_6$, phytic acid corresponds to a hexa-phosphoric acid ester of inosite plus 3 H_2O.

$$C_6H_6O_6[PO(OH)_2]_6+3H_2O.$$

At present it is impossible to say whether the compound represents a pyrophosphate or if the water is linked in some other way. That the acid contains 12 acid (OH) groups as expressed in the formula of Starkenstein, which would also be the case if it were a hexa-phosphoric acid ester of inosite, and not 18 (OH) groups as in the formula of Neuberg, seems certain, for in no case have we been able to prepare any salt in which more than 12 H-valences were replaced by bases.

As observed by Starkenstein only one half of the 12(OH) groups are particularly reactive. This finds expression in the fact that the barium salt obtained from acid solutions contains only 3 Ba to 6 P. As suggested by the above author, it is probable that these reactive hydroxyls are adjacent but linked to different phosphoric-acid residues. The salts with binary bases would then be represented by the following:



A further confirmation of this is found in the fact that the tri-barium phytate as well as other similar salts of phytic acid with binary bases are strongly acid in reaction.

The presence of only 8 acid (OH) groups, however, can be shown by titrating an aqueous solution of the acid with deci-normal sodium hydroxide using phenolphthalein as indicator Patten and Hart (loc. cit.) who titrated with deci-normal barium hydroxide using phenolphthalein as indicator obtained results agreeing with a hexa-barium salt.

Of special interest in connection with the constitution of phytin are the phosphoric acid esters of inosite.

Neuberg and Kretschner[1] report obtaining a poly-phosphoric acid ester of inosite by their method of preparing phosphoric acid esters of the carbohydrates and glycerine, that is, by the action of phosphorus oxychloride. The product could, however, not be obtained pure as it was found impossible to separate it from the inorganic phosphates.

[1] *Biochem. Ztschr.* **36**: 5.

Contardi[1] claims to have prepared the hexa-phosphoric acid ester of inosite by heating inosite with an excess of phosphoric acid in a stream of carbon dioxide to 160°–165° C. The product was purified as the barium salt and after decomposing the latter with sulphuric acid the free ester was obtained, which he describes as identical with phytic acid. The same author[2] claims to have prepared poly-phosphoric acid esters of mannite, quercite and glucose by the same method.

Carré,[3] however, repeating these experiments, found that the products described by Contardi were merely mixtures of free phosphoric acid and the polyhydric alcohols in question together with their decomposition products mixed with some monobarium phosphate.

Many fruitless efforts have been made in this laboratory to synthesize phytic acid and the hexa-phosphoric acid ester of inosite. All experiments in this direction lead only to the tetra-phosphoric acid ester of inosite, $C_6H_6(OH)_2\ O_4\ [PO(OH)_2]_4$.

The method of Contardi was modified to the extent that inosite, either dry or with water of crystallization, was heated with phosphoric acid, previously dried at 100° to constant weight, in vacuum to a temperature of 140°–160° C. for about two hours.

The same product, viz., the tetraphosphoric ester was obtained whether the phosphoric acid was present in large or small excess above six molecules of H_3PO_4 to one molecule of inosite. When it was present in less quantity than this, however, for instance 1 molecule of inosite to 3 molecules of H_3PO_4, then a mixture of esters was formed. It was found impossible to separate these products completely owing to the fact that they possess about the same solubility.

The tetraphosphoric ester is most conveniently isolated by means of its barium salt. The separation of the ester from the excess of the phosphoric acid or barium phosphate succeeded because its barium salt is much less soluble in dilute alcohol acidified with hydrochloric acid than is barium phosphate.

The new ester is a well characterized compound, very similar in appearance and reactions to phytic acid. By heating with acids, inosite and phosphoric acid are regenerated. It gives a white precipitate with the ordinary molybdate solution, and with excess of silver nitrate a white precipitate is also produced. These reactions are identical with those of phytic acid.

The inosite used in these experiments was prepared from the crude magnesium compound previously mentioned and carefully purified by recrystallization.

The reason why phytic acid could not be obtained by the action of phosphoric acid on inosite is no doubt to be found in that it is not a simple ester but a complex compound as suggested by Starken-

[1] *Atti R. Accad. dei. Lincei*, Roma (5) **19, I**: 23.
[2] *Atti R. Accad. dei. Lincei*, Roma (5) **19, I**: 823.
[3] *Bull. Soc. Chim. France* (4) **9**: 195.

stein. It is, however, difficult to understand why the hexa-phosphoric ester was not obtained by this method. The only explanation that can be offered is that under the conditions of these experiments it is not stable.

One reason alleged by Starkenstein for considering phytin a pyrophosphate is based upon its giving a white precipitate with silver nitrate. This is certainly a characteristic reacton of pyrophosphates. Yet the tetraphosphoric ester gives a pure white precipitate with the same reagent. As the ester cannot be in the form of a pyrophosphate the fact that phytic acid gives the same colored silver compound is not necessarily an indication that it represents a pyrophosphate compound.

The phytic acid used in these experiments was prepared from products obtained from two different sources. The starting material in one case was a calcium phytate imported from Europe; the other was a crude natural magnesium organic-phosphorus compound extracted in this country and kindly supplied us by Dr. Carl S. Miner of Chicago.

As shown by the analyses of the carefully purified salts and of the free acid, these two preparations were identical and they were also identical with the product described as phytic acid by Posternak and other investigators.

EXPERIMENTAL PART.

TRI-BARIUM PHYTATE.

The commercial phytin was purified for analysis by means of the barium salt. 30 gm. calcium phytate was dissolved in a small quantity of 0.5 per ct. hydrochloric acid, diluted to about 2 liters with water and a concentrated solution of 30 gm. barium chloride was added. The precipitate was dissolved without filtering by the addition of just sufficient dilute hydrochloric acid. It was then precipitated by adding barium hydroxide to faintly alkaline reaction. The mixture was then acidified with acetic acid and after standing over night was filtered and well washed in water. It was re-precipitated in the same manner three times. After finally filtering and washing in water the substance was dissolved in about 1 liter of 0.5 per ct. hydrochloric acid, filtered and the filtrate precipitated by adding a like volume of alcohol. After repeating this operation the substance was filtered, washed free of chlorides with 50 per ct. alcohol and finally washed in alcohol and ether and dried in vacuum over sulphuric acid.

The product so obtained was a light, perfectly white semi-crystalline or amorphous powder. Placed on moist litmus paper, it showed a strong acid reaction. It is very slightly soluble in water, slightly soluble in acetic acid and readily soluble in mineral acids.

For analysis the substance was dried at 130° C.

0.2728 gm. substance gave 0.0352 gm. H_2O and 0.0643 gm. CO_2.
0.2763 gm. " " 0.1749 gm. $BaSO_4$ and 0.1675 gm. $Mg_2P_2O_7$.
0.1909 gm. " " 0.1206 gm. $BaSO_4$ and 0.1154 gm. $Mg_2P_2O_7$.

For $C_6H_{12}O_9$ $[(PO_3H)_2\ Ba]_3$ = 1120.
Calculated C 6.42 per ct., H 1.60 per ct., P 16.60 per ct., Ba 36.78 per ct.
Found C 6.42 per ct., H 1.44 per ct., P 16.89 per ct., Ba 37.25 per ct.
P 16.85 per ct., Ba 37.17 per ct.

The barium salt prepared in the same manner from a natural crude magnesium organic phosphorus compound gave the following result on analysis:
0.2057 gm. substance gave 0.0273 gm. H_2O and 0.0480 gm. CO_2
0.1422 gm " " 0.0886 gm. $BaSO_4$ and 0.0841 gm. $Mg_2P_2O_7$.
Found C 6.36 per ct., H 1.48 per ct., P 16.48 per ct., Ba 36.66 per ct.
The two salts are therefore identical.

CRYSTALLIZED TRI-BARIUM PHYTATE.

One gm. purified phytic acid was dissolved in 10 cc. water and 4 gm. of the above mentioned tri-barium phytate added. It was filtered from traces of undissolved particles and allowed to stand for two days at room temperature. The substance had then separated as a heavy crystalline powder of irregular form. From less concentrated solutions the substance separates in small, needle-shaped crystals.

The substance was filtered, washed well in water and finally in alcohol and ether and dried in the air.

For analysis it was dried at 120° C.
0.1972 gm. substance lost 0.0153 gm. H_2O.
0.2028 gm. substance gave 0.1251 gm. $BaSO_4$ and 0.1216 gm. $Mg_2P_2O_7$.
Found P 16.71 per ct., Ba 36.30 per ct.
Calculated for $5H_2O$, 7.44 per ct. Found 7.75 per ct.

PENTA-BARIUM PHYTATE.

This salt is obtained on neutralizing a solution of the tri-barium phytate in 0.5 per ct. hydrochloric acid with barium hydroxide and then acidifying with acetic acid. The precipitate was filtered, washed thoroughly in water, alcohol and ether and dried in vacuum over sulphuric acid.

The product was a white amorphous powder. For analysis the substance was dried at 130° C.
0.2970 gm. substance gave 0.0307 gm. H_2O and 0.0500 gm. CO_2.
0.2507 gm. " " 0.2080 gm. $BaSO_4$ and 0.1207 gm. $Mg_2P_2O_7$.
0.1856 gm. " " 0.1543 gm. $BaSO_4$ and 0.0899 gm. $Mg_2P_2O_7$.
For $C_6H_{14}O_{27}P_6\ Ba_5$ = 1391.
Calculated C 5.17 per ct., H 1.00 per ct., P 13.37 per ct., Ba 49.37 per ct.
Found C 4.59 per ct., H 1.15 per ct., P 13.42 per ct., Ba 48.82 per ct.
P 13.50 per ct., Ba 48.92 per ct.

PENTA-BARIUM AMMONIUM PHYTATE.

When the tri-barium phytate is digested in dilute ammonia it is transformed into the penta-barium ammonium salt and ammonium

phytate. The latter product, however, was found to contain some barium.

Two gm. of the analyzed tri-barium phytate was digested for two hours in 25 cc. of 2.5 per ct. ammonia, filtered and washed in dilute ammonia and finally in alcohol and dried in vacuum over sulphuric acid. The product was a heavy white amorphous powder. On moist litmus paper it showed a neutral reaction.

For analysis the substance was dried at 130° C.

0.1509 gm. substance gave 0.1205 gm. $BaSO_4$ and 0.0762 gm. $Mg_2P_2O_7$.
0.1747 gm. " " 0.0026 gm. N (Kjeldahl*)

For $C_6H_{12}O_{27}P_6Ba_5(NH_4)_2 = 1425$.

Calculated P 13.05 per ct., Ba 48.19 per ct., N 1.96 per ct.
Found P 14.07 per ct., Ba 46.99 per ct., N 1.48 per ct.

By evaporating the filtrate from the above to dryness on the water-bath an amber-colored mass remained which after drying at 130° C. gave the following result on analysis:

Found P 20.51 per ct., Ba 6.65 per ct., N 10.48 per ct.

PENTA-MAGNESIUM AMMONIUM PHYTATE.

Two gm. phytic acid was dissolved in 400 cc. water and then precipitated by adding excess of magnesia mixture slowly and under constant shaking. After the precipitate had settled the supernatant liquid was decanted, the residue filtered and washed with water until free from chlorides and finally washed in alcohol and ether and dried in vacuum over sulphuric acid.

The product was a fine white amorphous powder and weighed 2.7 gm. It reacts neutral on moist litmus paper.

For analysis it was dried at 130°.

0.1089 gm. substance gave 0.0832 gm. $Mg_2P_2O_7$ for P.
0.1089 gm. " " 0.0705 gm. $Mg_2P_2O_7$ for Mg.
0.1248 gm. " " 0.0039 gm. N
0.0893 gm. " " 0.0028 gm. N } Kjeldahl)

For $C_6H_{12}O_{27}P_6Mg_5(NH_4)_2 = 859.5$.

Found P 21.29 per ct., Mg 14.13 per ct., N 3.12 per ct.—3.13 per ct.
Calculated P 21.64 per ct., Mg 14.13 per ct., N 3.25 per ct.

If the phytic acid is first neutralized with ammonia and then precipitated with magnesia mixture the same product is obtained.

Two gm. phytic acid in 400 cc. water was neutralized with ammonia, precipitated with excess of magnesia mixture, filtered, washed free of chlorides with dilute ammonia and then in alcohol and dried in vacuum over sulphuric acid.

For analysis the substance was dried at 130° C.

Found P 21.49 per ct., Mg 13.96 per ct., N 3.47 per ct., 3.48 per ct.

TETRA-CUPRIC DI-CALCIUM PHYTATE.

To a solution of 2 gm. phytic acid in 200 cc. water excess of calcium chloride was added and the solution then neutralized with ammonia.

* This and subsequent nitrogen determinations were made by Mr. M. P. Sweeney.

The precipitate was just dissolved in dilute hydrochloric acid and the solution precipitated with copper acetate. The bluish-green colored copper-compound was filtered off, washed with water until free from chlorides and then in alcohol and dried in vacuum over sulphuric acid.

The dry substance was a light blue amorphous powder. It is very slightly soluble in water or in very dilute acids, readily soluble in the ordinary dilute mineral acids. It is readily soluble in 2.5 per ct. ammonia with a deep blue color. In this solution concentrated ammonia or alcohol produces a light-blue colored precipitate.

The compound represents a nearly pure tetra-cupric di-calcium phytate. It contained 0.17 per ct. N.

For $C_6H_{12}O_9(PO_3Cu)_4 \cdot (PO_3Ca)_2 = 1036$.

Calculated Cu 24.51 per ct., Ca 7.72 per ct., P 17.95 per ct.

Found Cu 25.58 per ct., Ca 7.69 per ct., P 16.85 per ct.

If a slightly acid solution of magnesium ammonium phytate is precipitated with copper acetate a light blue colored copper compound is obtained. After washing and drying it gave the following result on analysis:

Mg 1.11 per ct., Cu 34.27 per ct., N 0.64 per ct., and 0.52 per ct., P 15.66 per ct.

This compound is exceedingly soluble in dilute and concentrated ammonia. By the careful addition of alcohol to the ammoniacal solution a substance separates in light blue colored crystals on standing. This is evidently a complex copper-ammonium salt but it was not further examined.

PHYTIC ACID.

This was prepared after the method of Patten and Hart (loc. cit.). The analyzed tri-barium salt was decomposed with the calculated quantity of deci-normal sulphuric acid. After removing the barium sulphate, the solution was precipitated with copper acetate. The copper compound was decomposed with hydrogen sulphide, the copper sulphide filtered off, the filtrate concentrated in vacuum and finally dried in vacuum over sulphuric acid. The products obtained from both the calcium phytate and the magnesium compound were light amber-colored, very thick liquids and corresponded in all respects with the body described by other investigators as phytic acid.

For analysis the substance was dried at 130° C.

a. From calcium phytate.

0.3193 gm. substance gave 0.0917 gm. H_2O and 0.1238 gm. CO_2.

0.1505 gm. " " 0.1424 gm. $Mg_2P_2O_7$.

b. From the magnesium compound.

0.2789 gm. substance gave 0.0804 gm. H_2O and 0.1101 gm. CO_2.

0.1236 gm. " " 0.1160 gm. $Mg_2P_2O_7$.

For $C_6H_{24}O_{27}P_6 = 714$.

Calculated.	Found a.	Found b.
C 10.08 per ct.	10.57 per ct.	10.76 per ct.
H 3.36 "	3.21 "	3.22 "
P 26.05 "	26.37 "	26.16 "

Titrated against deci-normal sodium hydroxide using phenolphthalein as indicator the following results were obtained:

0 2648 gm. acid required 30.7 cc. N/10 NaOH.
Calculated for 8 NaOH 29.65 cc.

0.1593 gm. acid required 18.60 cc. N/10 NaOH.
Calculated for 8 NaOH 17.60 cc.

INOSITE FROM THE CRUDE MAGNESIUM COMPOUND.

Twenty-five gms. of the air-dried substance, containing 20 per ct. of moisture, was heated with 100 cc. 30 per ct. sulphuric acid in a sealed tube for about three hours at a temperature of 140° C. Two tubes equally charged were heated at the same time. After cooling, the reaction mixture was of dark brown color and a considerable quantity of magnesium salts had crystallized out.

The contents were washed into a beaker, filtered and diluted with water to about 1500 cc. The sulphuric and phosphoric acids and the magnesium were then precipitated by barium hydroxide, filtered and well washed in hot water. The filtrate was evaporated to about 350 cc. and the excess of barium removed by carbon dioxide, filtered, the filtrate decolorized with animal charcoal and then evaporated on the water bath to a syrupy consistency. This was taken up in a small quantity of hot water, filtered and alcohol added to the filtrate until a cloudiness was produced. By scratching with a glass rod crystallization began; more alcohol was then added and the mixture placed in the ice-chest over night. After filtering and washing in alcohol and ether and drying in the air the product weighed from 5.1 to 5.4 gm. From the mother liquor a further quantity of crystals from 0.4 to 0.6 gm. could be obtained on the addition of ether and allowing to stand for twenty-four hours in the cold.

For purification the raw product was dissolved in 6 parts of water and again brought to crystallization by the addition of alcohol as before. It was then obtained in large, thin, colorless plates.

It gave the reaction of Scherer. The dried substance melted at 220° C. (uncor.)

Dried at 100° C. 0.4136 gm. substance lost 0.0669 gm. H_2O and 0.1600 gm. lost 0.0258 gm. H_2O.

The dried substance was analyzed.

0.1342 gm. substance gave 0.0791 gm. H_2O and 0.1981 gm. CO_2.

For $C_6H_6(OH)_6 = 180$.

Calculated C 40.00 per ct., H 6.66 per ct., 2 H_2O 16.66 per ct.
Found C 40.26 per ct., H 6. 59 per ct., 2 H_2O 16.17 per ct. – 19.12 per ct.

This substance was used in subsequent experiments with phosphoric acid. Some 40 gm. of inosite were prepared in this way.

TETRA-PHOSPHORIC ACID ESTER OF INOSITE.

4.32 gm. (2 mol.) crystallized inosite was powdered and mixed in a distillation flask with 24 gm. phosphoric acid (about 24 mol. or double the quantity required to form the hexa-phosphoric ester). The acid had been previously dried at 100° C. to constant weight. The flask was connected with the vacuum pump and heated in an oil bath to 140°–160° C. for about two hours. By 120° water began to come over and the reaction was practically complete at the end of one hour. After cooling, the reaction mixture was a thick, reddish-brown colored, nearly solid mass. This was dissolved in about 1 liter of water and a solution of 40 gms. of barium chloride in 400 cc. of water was added. The barium salt of the ester was then precipitated by the addition of about 1 liter of alcohol.

A solution containing phosphoric acid and barium chloride in the same dilution as above remains perfectly soluble on the addition of a like volume of alcohol.

The voluminous flaky precipitate was filtered off at once and thoroughly washed in $33\frac{1}{3}$ per ct. alcohol.

For purification the substance was dissolved in 700 cc. of 0.5 per ct. hydrochloric acid, filtered from slight insoluble residue, the filtrate diluted with 500 cc. of water, some barium chloride added and then precipitated by the addition of a like volume of alcohol. This was repeated a second time. The substance was then dissolved in 500 cc. of 0.5 per ct. hydrochloric acid, precipitated by adding barium hydroxide to slightly alkaline reaction, then acidifying with hydrochloric acid and adding 500 cc. alcohol. After filtering and washing as before the substance was again twice precipitated from 0.5 per ct. hydrochloric-acid solution with alcohol and finally washed in 50 per ct. alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product weighed 8.9 gm. It was a white voluminous amorphous powder. On moist litmus paper it showed a strong acid reaction. The solubility of the product was practically the same as for the tri-barium phytate.

For analysis it was dried at 100° and 130° C.

0.3252 gm. substance lost 0.0281 gm. H_2O.

0.2697 gm. substance gave 0.0442 gm. H_2O and 0.0878 gm. CO_2.
0.2038 gm. " " 0.0300 gm. H_2O " 0.0685 gm. CO_2.
0.2482 gm. " " 0.1505 gm. $BaSO_4$ " 0.1434 gm. $Mg_2P_2O_7$.
0.1833 gm. " " 0.1108 gm. $BaSO_4$ " 0.1075 gm. $Mg_2P_2O_7$.
0.1776 gm. " " 0.1074 gm. $BaSO_4$ " 0.1038 gm. $Mg_2P_2O_7$.

For $C_6H_6(OH)_2O_4\,[(PO_3H)_2Ba]_2 = 770.7$.

Calcul'd C 9.34 per ct., H 1.55 per ct., P 16.08 per ct., Ba 35.64 per ct.
Found C 8.87 per ct., H 1.83 per ct., P 16.10 per ct., Ba 35.68 per ct.
C 9.16 per ct., H 1.64 per ct., P 16.34 per ct., Ba 35.57 per ct.
P 16.29 per ct., Ba 35.58 per ct.

Calculated for 4 H_2O, 8.55 per ct. Found 8.64 per ct.

Another lot prepared by heating 1.80 gm. dry inosite (1 mol.) with 7.9 gm. dry phosphoric acid (about 8 mol.) and isolated in the same manner gave the following results on analysis:

0.2879 gm. substance lost 0.0240 gm. H_2O.

The dried substance was analyzed.
0.2639 gm. substance gave 0.0452 gm. H_2O and 0.0936 gm. CO_2.
0.1480 gm. " " 0.0866 gm. $BaSO_4$ " 0.0846 gm. $Mg_2P_2O_7$.
0.1632 gm. " " 0.0959 gm. $BaSO_4$ " 0.0933 gm. $Mg_2P_2O_7$.

Found C 9.67 per ct., H 1.91 per ct., P 15.93 per ct., Ba 34.43 per ct.
H_2O 8.33 per ct., P 15.93 per ct., Ba 34.58 per ct.

A third lot prepared by heating 1.80 gm. dry inosite (1 mol.) with 5.88 gm. dry phosphoric acid (6 mol.) and isolating in the same manner as before gave the following:
C 9.69 per ct., H 1.75 per ct., P 16.06 per ct., Ba 36.33 per ct.

It is apparent therefore that in each of the above experiments the same compound was produced.

THE FREE TETRA-PHOSPHORIC ESTER.

About 5 gm. of the purified barium salt was decomposed by digesting it with the calculated quantity of deci-normal sulphuric acid. After removing the barium sulphate the solution was precipitated with excess of copper acetate. The copper precipitate was filtered, thoroughly washed with water, suspended in water and decomposed with hydrogen sulphide. The copper sulphide was removed by filtration, the filtrate concentrated in vacuum and finally dried in vacuum over sulphuric acid until it was of a thick, syrupy consistency.

For analysis the substance was dried at 130° C.
0.3020 gm. substance gave 0.0933 gm. H_2O and 0.1577 gm. CO_2.
0.1605 gm. " " 0.1387 gm. $Mg_2P_2O_7$.

For $C_6H_6(OH)_2O_4[PO(OH)_2]_4 = 500$.

Calculated C 14.40 per ct., H 3.20 per ct., P 24.80 per ct.
Found C 14.24 per ct., H 3.45 per ct., P 24.09 per ct.

0.1663 gm. substance required 16.5 cc. deci-normal sodium hydroxide using phenolphthalein as indicator. This corresponds to 5 acid (OH) groups.
Calculated for 5 NaOH 16.63 cc.

PROPERTIES OF THE FREE ESTER.

The concentrated aqueous solution of the ester is very similar to phytic acid. It is a very thick, light, amber-colored liquid of sharp acid, slightly astringent taste and strong acid reaction. On longer keeping in the desicator over sulphuric acid it becomes hard and brittle and may be powdered. It is then very hygroscopic.

The dry substance is slowly but completely soluble in alcohol, readily soluble in water.

The concentrated aqueous solution gives a white precipitate with silver nitrate in excess which dissolves on largely diluting with water. The precipitate is readily soluble in ammonia, dilute nitric, sulphuric and acetic acids, insoluble in glacial acetic acid.

With ferric chloride it gives a white or faintly yellowish precipitate which is very sparingly soluble in acids.

With lead acetate a white precipitate is produced, readily soluble in dilute nitric acid, but sparingly soluble in acetic acid.

With barium chloride it gives a white precipitate slightly soluble in acetic acid, but readily soluble in hydrochloric and nitric acids.

Calcium chloride does not give a precipitate, but, on heating, the calcium salt is thrown down as a white precipitate which redissolves on cooling.

Magnesium salts do not cause a precipitate, and on heating the solution merely turns cloudy; on cooling it clears up again.

With the ordinary molybdate solution it gives in the cold a white voluminous flaky precipitate which slowly turns yellowish in color. Phytic acid under the same conditions gives a white precipitate which remains unchanged in the cold. On drying at 110° or 130° the substance turns very dark in color.

The ester, like phytic acid, fails to give directly the Scherer reaction for inosite.

INOSITE FROM THE TETRA-PHOSPHORIC ESTER.

Ten gm. of the purified barium salt was heated with 25 cc. 30 per ct. sulphuric acid in a sealed tube to about 150° C. for three hours. After precipitating the sulphuric and phosphoric acids with barium hydroxide the inosite was isolated by the usual method and recrystallized from hot dilute alcohol. It was filtered and washed in alcohol and ether and dried in the air. Yield 1.52 gm. It was obtained in the form of small colorless six-sided plates, free from water or crystallization. The air-dried, water-free substance melted at 221° C., uncor.

0.2094 gm. substance gave 0.1259 gm. H_2O and 0.3033 gm. CO_2.
0.1360 gm. " " 0.0827 gm. H_2O " 0.1991 gm. CO_2.

For $C_6H_{12}O_6 = 180$.

Calculated	C 40.00 per ct., H 6.66 per ct.
Found	C 39.50 per ct., H 6.72 per ct.
	C 39.93 per ct., H 6.80 per ct.

As already mentioned, if a mixture of inosite and phosphoric acid is heated when less than 6 mol. H_3PO_4 are present to 1 mol. inosite, a mixture of esters is obtained. It was found impossible to separate these bodies as barium salts and obtain pure compounds, since their solubilities are apparently nearly alike.

3.60 gm. dry inosite (2 mol.) and 5.88 gm. dry phosphoric acid (6 mol.) were heated in a distillation flash as before to 180°–190° for

about two hours, until water ceased coming over. The reaction mixture was in form of a very bulky, thin flaky mass, very brittle and of yellowish-brown color, mixed with some very dark-colored substance. It was broken up with a glass rod and removed from the flask and treated with water, in which the dark-colored portion was readily soluble, but the lighter-colored substance was insoluble in this medium. It was powdered in a mortar and thoroughly washed in water and alcohol and dried in vacuum over sulphuric acid.

The substance was apparently insoluble in boiling water, in boiling dilute acids and in glacial acetic acid; also insoluble in alcohol, ether and other organic solvents. After drying at 130° the substance was analyzed.

0.2500 gm. substance gave 0.0838 gm. H_2O and 0.2085 gm. CO_2.
0.1500 gm. " " 0.1145 gm. $Mg_2P_2O_7$.
0.1542 gm. " " 0.1178 gm. $Mg_2P_2O_7$.

Found C 22.74 per ct., H 3.75 per ct., P 21.28 per ct., 21.29 per ct.

This agrees approximately with a mono-pyro-phosphoric ester of inosite, but the phosphorus is too high.

It was decided to purify it by means of the barium salt. The substance was dissolved by boiling in dilute sodium hydroxide, in which it gave a dark amber-colored solution. After filtering it was precipitated with barium chloride; the barium precipitate filtered and washed free of alkali. It was then dissolved in 500 cc. 0.5 per ct. hydrochloric acid and precipitated by barium hydroxide. After filtering and washing it was repeatedly precipitated with alcohol from 0.5 per ct. hydrochloric-acid solution until finally a small amount of a white amorphous powder was obtained. After drying at 130° this was analyzed.

0.2028 gm. substance gave 0.0412 gm. H_2O and 0.0979 gm. CO_2.
0.2207 gm. " " 0.0413 gm. H_2O " 0.1042 gm. CO_2.
0.1982 gm. " " 0.0996 gm. $BaSO_4$ " 0.1103 gm. $Mg_2P_2O_7$.

Found C 13.16 per ct., H 2.27 per ct., P 15.51 per ct., Ba 29.57 per ct.
C 12.88 per ct., H 2.09 per ct.

In this compound the relation between the carbon and phosphorus is nearly 6 C to 3 P, which would indicate a tri-phosphoric ester. The substance was, however, far from pure, and lack of material prevented any further investigation of this body, which is apparently a mixture of various esters.

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TECHNICAL BULLETIN No. 20 MAY, 1912

New York Agricultural Experiment Station.

GENEVA, N. Y.

A STUDY OF THE METABOLISM AND PHYSIOLOGICAL EFFECTS OF CERTAIN PHOSPHORUS COMPOUNDS WITH MILCH COWS, II

A. R. ROSE

PUBLISHED BY THE DEPARTMENT OF AGRICULTURE

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TECHNICAL BULLETIN No. 20.

A STUDY OF THE METABOLISM AND PHYSIOLOGICAL EFFECTS OF CERTAIN PHOSPHORUS COMPOUNDS WITH MILCH COWS, II.

A. R. ROSE.

SUMMARY.

The chief aim of this experiment was to check the results reported in Technical Bulletin No. 1, of this Station, by repeating the work in such a way as to eliminate more of the variable factors. This was effected by adjusting one of the animals used in the previous experiments to a low-phosphorus ration very nearly identical with the one formerly employed and adding thereto the calcium salt of phytin.

In this, as in the former experiments, the organic phosphorus ingested was eliminated very largely in the form of inorganic phosphorus by way of the intestine, the amounts of phosphorus in the urine being very small. When phytin was withdrawn from the ration, the decrease of phosphorus in the urine was immediate; when phytin was added, a rise in phosphorus occurred after a lag of two days. Phytin caused more phosphorus to be eliminated through the kidney than did whole wheat bran. The long duration of the low-phosphorus period did not in itself affect the phosphorus content of the urine nor the phosphorus balance.

The insoluble phosphorus of the feces diminished with decreasing amounts of insoluble phosphorus in the rations, when the latter ranged above fourteen grams.

The soluble organic phosphorus disappeared very largely from the alimentary tract. The apparent utilization was poorer in the low-phosphorus periods and in the calcium phytate period than in the whole-bran period.

For maintenance of phosphorus equilibrium in this species of animal the requirement would seem to be the amount of phosphorus eliminated in the milk plus twenty-six milligrams per kilo of body weight; an excess over this amount causes phosphorus retention, and smaller quantities result in loss of phosphorus from the organism.

The addition of calcium phytate increased the potassium both in the urine and dung, and changed the path of elimination of part of the magnesium from the kidney to the intestine. The calcium added as calcium phytate was almost entirely eliminated by the intestine immediately after administration. The calcium of the urine increased with decreasing phosphorus in the rations and decreased when calcium phytate was added.

The nitrogen compounds of the ration were well utilized and for the most part a positive nitrogen balance was maintained. The animal gained 19 kilos during the experiment, half of which could be accounted for by the plus balance of nitrogen. There was a suggestion of a parallelism between the nitrogen and phosphorus balances.

The former observations as to the influence of phosphorus compounds on the oestrum and the amount of urine voided were not corroborated; neither was the laxative effect previously noted. The difference in the moisture content of the feces of the several periods of this experiment was very small.

A long low-phosphorus period resulted in unfavorable symptoms. The animal returned to a normal condition after a week's feeding on ash-rich rations including alfalfa, silage and wheat bran.

The volume of the milk fluctuated inversely with the amount of phytin phosphorus in the rations. The increase of milk flow on removal of phytin was not a mere dilution. Except for the change in the amount of fat, the composition of the milk was not materially altered. The responses of the fat to the fluctuations of phytin phosphorus were immediate and consistent, as distinct, though not quite as large, as in the previous experiments. The best milk flow, both as to amount and fat content, happened to occur in the period of phosphorus equilibrium.

INTRODUCTION.

The work reported in this bulletin is the fourth experiment in a series planned by Director Jordan in 1904, for the purpose of ascertaining the specific influence of the ash constituents of plants upon animal metabolism and especially to learn what effect these very important elements may have on milk production. As biochemical research progresses, we have come to realize more and more the very important part which the ash constituents play in all process of life. Agricultural chemists have given much attention to phosphorus, and this important substance has become familiar to farmers. It was naturally, therefore, the first element to be studied in this undertaking, and this substance still holds the chief interest of those carrying out Director Jordan's comprehensive program.

The first task of the investigators was to find out the nature of phosphorus in the grains and forage plants usually employed in feeding cattle. This work was published as bulletins 238 and 250 of this Station by Hart and Andrews, and Patten and Hart, respectively.[1] In these bulletins it is shown that the phosphorus of the grains is very largely in the form of a soluble organic compound,

[1] Hart and Andrews, N. Y. Agr. Expt. Sta. Bull. No. 238.
Patten and Hart, N. Y. Agr. Expt. Sta. Bull. No. 250.

previously described and named by the Swiss chemist, Posternak.[1] Phytin, as this compound is called, is in itself a very interesting substance; but its physical and chemical properties will not be discussed in this report. For descriptions of phytin, the reader is referred to the bulletins mentioned above and the paper by Posternak. The author wishes, however, to make a correction with respect to the constitution of phytin as given in these bulletins. The more recent work[2] has demonstrated that its acid is not anhydrooxymethylenediphosphoric acid as stated by Posternak and accepted by Hart at the time of publication of his work, but is an inositephosphoric acid. Recent work by Anderson[3] carried on in the laboratory of this Station confirms this later view of the chemical constitution of phytin.

PREVIOUS EXPERIMENTS.

The role of phosphorus in metabolism has been a live question for a long time and a large mass of data has been accumulated regarding it. A number of important facts are definitely established and others have been suggested. Since these have been summarized by Jordan, Hart, and Patten[4] and more recently reviewed in detail by Albau and Neuberg,[5] the reader is referred to these and only those references will be made which bear on special points as the discussion progresses. Technical Bulletin No. 1 of this Station reports the first three feeding experiments in this series and is a valuable contribution to our knowledge of mineral metabolism. The chief component of the rations used was wheat bran, chosen because of its relatively high content of phytin, which can be easily removed by leaching, the other constituents contributing but a very small amount of phosphorus. By alternating periods of rations containing washed and unwashed bran, a marked contrast in the amount of administered phosphorus was produced. This ranged from thirteen to sixteen grams in the low phosphorus period and from seventy-seven to eighty-one grams in the high phosphorus period. Excepting the variations in calcium, magnesium, and potassium, the other conditions were kept as constant as is practicable in experiments of this kind. A large number of observations were made and from the mass of analytical data the authors drew conclusions which may be briefly summed up as follows:

Increase of phosphorus in the ration increases the phosphorus elimination;

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[1] Posternak; *Rev. Gen. Bot.* **12:** 5, 65 (1900); *Compt. Rend. Soc. Biol.* **55:** 1190 (1902); *Compt. Rend.* **137:** 202, 337, 439 (1903); **140:** 322 (1905).

[2] Suzuki, Yoshimura, Takaishi. Bul. Coll. Agr. Tokio, **7:** 495, 503, 1907. Neuberg; *Biochem. Ztschr.* **9:** 557 (1908); Starkenstein; *Biochem. Ztschr.* **30:** 56 (1910).

[3] Anderson, R. J. N. Y. Agrl. Expt. Sta. Tech. Bul. No. 19.

[4] Jordan, Hart and Patten. N. Y. Agr. Expt. Sta. Tech. Bull. No. 1.

[5] Albau and Neuberg. Physiologie und Pathologie des Mineralstoffwechsels.

Increase of organic phosphorus in the ration causes an increased elimination of inorganic phosphorus, the quantity of outgoing organic phosphorus being but slightly affected by the intake of organic phosphorus.

Increased phosphorus elimination is, in the herbivora, mostly by way of the intestines.

A large intake of phosphorus causes a retention of this element.

When the phosphorus given is insufficient in quantity the organism uses for its normal functions the phosphorus previously stored in the body, or that which is not serving an immediately vital purpose.

These results substantiate work done in other laboratories.

The following results which were also noted suggest other possible conclusions. These, if verified, would constitute new contributions to our knowledge and are of fundamental interest:

Sudden withdrawal of phosphorus from the ration causes dryer and firmer feces, sometimes constipation.

The volume of the urine varies directly with the quantity of phosphorus insoluble in 0.2 per ct. hydrochloric acid, and indirectly with the phosphorus soluble in this reagent.

The milk flow increases with the withdrawal of phosphorus and decreases when phosphorus is added to the ration.

Increase of phosphorus in the ration increases the fat in the milk, and vice versa.

These results were pronounced and consistent and must therefore be due to the differences produced in the wheat bran by leaching it with water. By this process, several substances were removed from the wheat bran; some protein, a small amount of carbohydrate, magnesium, potassium and phosphorus. The loss in the protein was made good by an equivalent amount in the form of wheat gluten; that in carbohydrate was deemed negligible. The largest differences were in the amounts of magnesium, potassium and phosphorus. Of these variable factors in the experiment, the phosphorus, in the form of phytin, was thought to be the most significant; the magnesium and calcium are probably combined with the phosphorus in the phytin. Following this suggestive clue, the plans so developed as to include other experiments in which the phosphorus would be made more definitely the variable factor by adding some salt or combination of salts of phytin to a basal ration of a very low phosphorus content, and thus discover specifically what forms of phosphorus, or combinations of bases with phosphorus, are responsible for these striking physiological effects. Inasmuch as calcium phytate can be bought in the market, and is also a single basic salt and therefore presents a simpler problem than the double salts, offering only two variable elements, it was chosen for the next experiment, which is the subject of the present report.

FOURTH EXPERIMENT.

PLAN.

The cow used in experiment II proved to be a hardy animal, a good milker, and hearty eater, by far the most suitable animal in the herd, and was therefore chosen as the subject of this experiment. When the work began she weighed 495 Ko. and gave nine and one-half kilos of milk. She did not come in regularly, but aborted on February 7. By April 11 she had been adjusted to the following ration: oat straw 4,536 grams, rice meal 2,724 grams, wheat bran 4,536 grams, wheat gluten 597 grams, which gives a total of 249.6 grams nitrogen, and 66.5 grams of phosphorus, 51.1 grams of the latter being in the form of phytin phosphorus. She was then removed from the herd and placed in a comfortable room planned for metabolism work, and after eighteen days on the ration as given above, samples for analysis were taken daily. The days of the experiment were numbered consecutively from the first day of sampling, April 29, to the end of the experiment, eighty-five days later. In the laboratory the samples were known by these numbers. The time was divided into five main periods with transition periods between each two as follows:

Period I, days 1–6 (April 29–May 4), The whole wheat bran period, with the ration as specified above;

Transition period, days 7–10, in which the whole bran was gradually replaced by washed bran;

Period II, days 11–19 (May 9–May 17), The phosphorus equilibrium period, in which the intake and outgo of phosphorus were approximately the same, 24.2 grams per day.

Period III, days 23–33 (May 21–May 31), The low phosphorus period in which the phosphorus was reduced to fifteen grams;

Transition period, on the thirty-fourth day, 50 grams of calcium phytate were added to this ration, this salt was increased to 125 grams on the next day;

Period IV, days 36–45 (June 3–June 12), The calcium phytate period, which was the same as the preceding period (III) plus one hundred and seventy-five grams of calcium phytate;

Transition period, days 46–50, in which decreasing amounts of the phytate were added to the ration;

Period V, days 51–78 (June 18–July 15), The protracted low-phosphorus period in which the ration was the same as that offered in Period III.

The plan included a sixth period identical with the first, in which striking results were anticipated as consequence of the change from the long continued low-phosphorus ration to one high in this element, but pathological conditions developed and the animal was perforce discharged on the eighty-sixth day.

In this plan we think we have maintained as nearly as possible constant conditions with respect to all factors involved, except the soluble bran constituents which were purposely changed at the end of the first and fifth periods and the addition of calcium phytate in Period IV. The salient points in the plan of the experiments are: a large decrease of total phosphorus, including soluble organic phosphorus and inorganic phosphorus; a decrease of magnesium and of potassium in the rations at the end of the first period, and an increase of calcium and phosphorus during the fourth period. This made the first and fourth periods high in phosphorus intake, and the others low, and the fourth distinguished from the first by having calcium phytate added to a ration, otherwise low in phosphorus.

THE EXPERIMENT IN PROGRESS.

In regard to the details, the management of the experiment was as like that reported in Technical Bulletin No. 1 of this Station, page 5 et seq., as it could practically be made. The division between the days was made at seven o'clock in the morning. The cow was milked twice a day instead of three times. The attendants for the cow were the same men as were employed for this purpose in the previous experiments.

The components of the rations were separately mixed and weighed into paper bags, at which time the samples were taken. The oat straw was a baled article purchased in the local market. Two separate batches of straw were used, the first (F6) lasting from April 11 to June 2, the other (F14) from June 3 to the end of the experiment. These differed somewhat in their analysis, as is shown in Table 1. Large quantities of wheat bran were extracted with water and dried (F5). The original bran (F2) contained 1.48 per ct. of total phosphorus, of which 73 per ct. of the phosphorus was soluble in 0.2 per ct. hydrochloric acid; the washed bran (F5) used in the second period had a total phosphorus content of 0.326 per ct. This latter bran was later rewashed (F11, F12, F13) and the phosphorus reduced to only 0.123 per ct.; this was used in the last three periods. The rice was of good quality, well polished and therefore low in ash constituents. It was ground in a local mill and weighed up at three different times (F7, F9, F15). The analyses of the samples agreed well with one another, and the values used are an average of the three. The wheat gluten was weighed up from two lots (F10, F36), the latter being introduced in the fifth period. It contained a little more moisture and calcium phosphate but did not differ enough from the first to affect the problem materially. The largest discrepancies introduced by the renewal of the components of the rations are those in the straw, which in terms of grams per day are: Total phosphorus 0.63, soluble phosphorus 0.36, inorganic phosphorus 1.45.

TABLE I.— MINERAL CONSTITUENTS OF FEEDS USED.

FEED.	Amount	Nitrogen.	PHOSPHORUS.			Calcium.	Magnesium.	Potassium.
			Total.	Soluble.	Inorganic.			
	Grams.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Straw (F 6).....	4,536	0.40	0.122	0.085	0.045	0.41	0.153	0.954
(F 14)....	4,536	0.38	0.108	0.093	0.077	0.35	0.150	0.941
Rice.....	2,724	1.25	0.083	0.036	0.014	0.09	0.020	0.025
Gluten* (F 10)..	.....	13.25	0.220	0.094	0.034	0.38	0.050	0.043
(F 36)..	.....	12.52	0.196	0.055	0.040	0.20	0.033	0.030
Bran (F 2).....	4,536	2.60	1.485	1.080	0.014	0.14	0.540	1.380
(F 5).....	4,536	2.02	0.326	0.112	0.056	.....	.....	.....
(F 12).....	4,536	1.89	0.123	0.039	0.005	0.37	0.080	0.075
Phytin.....	175	.....	20.32	20.32	2.00	11.87	0.550	.....

* Fed in period I 597 grams, II, III, IV, 796 grams, V 530 grams.

The animal was given plenty of protein and carbohydrate and ate her rations well until the seventeenth day, when she began to refuse varying amounts of feeds which were carefully gathered up and analyzed, and the daily ration corrected accordingly. The actual intake of the elements studied is given in Table II. This

TABLE II.— NITROGEN AND PHOSPHORUS INGESTED.

PERIOD.	Days		AVERAGE DAILY CONSUMPTION OF					
			Nitrogen total.	Phosphorus.				
				Total.	Soluble.	Insoluble	Phytin.	Inorganic.
			Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
I.....	1–6	April 29 to May 1	249.7	76.5	54.4	22.1	51.1	3.3
II.....	11–14	May 9	249.3	24.2	10.7	13.5	5.4	5.3
	13–17	to	247.2	24.0	10.6	13.4	5.4	5.2
	16–19	May 17	246.0	23.9	10.5	13.4	5.3	5.2
III.....	25–29	May 23	215.6	12.6	5.9	6.7	3.6	2.3
	27–31	to	192.0	12.6	5.8	6.8	3.4	2.4
	29–32	May 30	213.3	13.4	6.3	7.1	3.7	2.6
IV.....	39–42	June 6	184.0	41.6	39.2	2.4	32.7	6.5
	42–45	to	179.0	46.1	42.2	3.9	35.3	6.9
	36–45	June 12	190.3	43.8	40.0	3.8	32.7	6.9
V.....	51–56	.....	201.8	13.6	7.2	5.9	2.6	4.0
	59–64	June 26	167.2	11.0	5.4	5.6	2.7	3.7
	66–71	to	184.2	11.7	6.7	5.0	2.8	3.9
	72–77	July 14	193.3	13.0	7.0	6.0	3.2	3.9

refusal of part of the ration may have been due to an excess over her requirements and desires but was more probably due to her dislike for the washed bran and rice, and in the fourth and fifth periods to physiological disturbances. By the end of the fourth period she left such large quantities untouched that it was deemed advisable to decrease some of the rations.

COLLECTION AND TABULATION OF DATA.

The methods of sampling and analysis were the same as those given in Technical Bulletin No. 1. There were altogether 1,998 analyses recorded, mostly done in duplicate and a few in triplicate. The greatest number of these analyses were made by M. P. Sweeney and J. T. Cusick. This mass of data was tabulated, and in order to facilitate interpretation of results, groups of days have been averaged. These days were chosen before any study of the material had been made in order to eliminate any partiality that might unconsciously creep in.

The cow was kept for eighteen days on the first ration so as to become fully adjusted to it, and the figures for the six following days were averaged as Period I to represent the normal status of the animal. The ration planned to give phosphorus equilibrium covered thirteen days, the last nine of which were divided into three groups. The days of the first low-phosphorus period, also thirteen, were grouped in the same manner. To this ration calcium phytate was

TABLE III.—APPARENT DIGESTIBILITY* OF NITROGEN AND PHOSPHORUS.

Days		Nitrogen.		Phosphorus.					
				Total		Insoluble		Phytin	
		Grams.	*Per ct.*	*Grams.*	*Per ct.*	*Grams.*	*Per ct.*	*Grams.*	*Per ct.*
I	1–6	163.0 9.3	65.2	25.9	33.9	11.9	54.2	47.7	93.5
II	11–14	179.3	61.2	10.1	41.8	7.0	49.8	3.6	66.6
	13–17	181.8	61.3	11.7	48.9	6.8	50.0	5.2	96.3
	16–19	170.7	60.9	11.2	46.9	6.7	50.0	3.9	75.1
III	25–29	157.5	73.1	4.4	35.1	2.7	40.3	2.7	75.0
	27–31	131.5	68.5	3.8	30.0	1.3	19.1	2.5	73.9
	29–32	158.2	74.1	5.5	41.2	2.3	32.4	2.3	75.9
IV	39–42	135.2	73.4	24.1	58.0	(—3.1)	.....	33.0	91.1
	42–45	118.5	66.2	22.7	49.2	(—2.8)	.....	31.3	80.0
	36–45	133.1	70.0	24.6	57.2	(—2.7)	.....	31.8	86.7
V	59–64	113.8	67.6	3.2	29.0	1.6	29.8	0.0	0.0
	66–71	121.0	65.6	2.9	24.9	1.6	23.8	1.3	46.5
	72–77	123.6	64.0	4.0	30.0	0.4	7.6	1.6	50.0

* The difference between the amounts of nitrogen and phosphorus in the feces and the rations consumed.

added on the days Nos. 34 to 50, 50 grams on the 34th, 125 grams on the 35th, and 175 grams on each succeeding day until the 50th day, after which the amounts were gradually decreased. Four days were chosen in the middle and four days at the end of this period. Inasmuch as this period (IV) is the main feature of the experiment, an average is also taken of the entire time when the full amount of calcium phytate was added (days 36 to 45). The second low-phosphorus period was prolonged to show the effects of an exaggerated case of phosphorus starvation, and lasted from the 51st day to the 77th day. The average of days 51 to 56 is given to show the first changes induced by the withholding of the phosphorus from the ration, the other groups of days selected were three of six days each at the end of the period. These averages of the days of the several periods are presented in the main tables.

TABLE IV.— INTAKE AND OUTGO OF NITROGEN AND PHOSPHORUS.

A.—NITROGEN.

Period.	Days.	Intake.	Outgo.				Balance.
			Dung.	Urine.	Milk.	Total.	
		Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
I.....	1–6	249.6	86.6	92.9	58.7	238.2	+11.4
II.....	11–14	249.7	70.4	103.2	57.7	231.3	+18.4
	13–17	247.0	65.2	107.8	57.8	230.8	+16.2
	16–19	246.0	75.3	103.9	57.8	236.5	+9.5
III.....	25–29	215.6	58.1	101.1	56.7	215.9	—0.3
	27–31	192.0	60.5	106.8	54.1	221.4	—29.4
	29–32	213.3	55.1	97.8	55.4	208.3	+5.0
IV.....	39–42	184.0	48.8	80.8	46.3	175.9	+8.1
	42–45	179.0	60.5	72.5	48.5	181.5	—3.5
	36–45	190.3	57.2	83.9	50.6	191.7	—1.4
	51–56	201.8	65.3	67.3	54.1	186.7	+15.1
V.....	59–64	167.3	53.5	69.1	45.3	168.	—0.7
	66–71	184.3	63.3	73.6	45.4	182.3	+2.0
	72–77	193.3	69.7	66.2	45.6	181.5	+11.8

B.—TOTAL PHOSPHORUS.

I.....	1–6	76.5	50.6	2.2	9.5	62.2	+14.2
II.....	11–14	24.2	14.1	0.30	10.6	25.0	—0.8
	13–17	24.0	12.3	0.18	10.8	23.3	+0.7
	16–19	23.9	12.7	0.17	10.8	23.7	+0.2

TABLE IV.—INTAKE AND OUTGO OF NITROGEN AND PHOSPHORUS—*Continued*

B.—TOTAL PHOSPHORUS—*Continued.*

Period.	Days.	Intake.	Outgo.				Balance.
			Dung.	Urine.	Milk.	Total.	
		Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
III.....	25–29	12.6	8.2	0.13	10.5	18.9	—6.3
	27–31	12.6	8.8	0.13	10.5	19.4	—6.8
	29–32	13.4	7.9	0.13	10.3	18.3	—4.9
IV.....	39–42	41.5	17.4	5.3	9.2	31.9	+9.7
	42–45	46.1	23.4	5.7	9.4	38.5	+7.6
	36–45	43.8	19.2	4.0	9.7	32.9	+10.9
	51–56	13.6	10.1	0.11	10.3	20.5	—6.9
V.....	59–64	11.0	7.8	0.15	8.4	16.4	—5.4
	66–71	11.7	8.8	0.11	8.8	17.7	—6.0
	72–77	13.0	9.0	0.18	8.7	17.9	—4.9
		C.—SOLUBLE PHOSPHORUS.					
I.....	1–6	54.4	40.4	2.2	7.0	49.6	+4.8
II.....	11–14	10.7	7.6	0.30	8.2	16.1	—5.4
	13–17	10.6	5.5	0.18	8.5	14.18	—3.6
	16–19	10.5	6.0	0.17	8.4	14.57	—4.1
III.....	25–29	5.9	3.2	0.13	8.3	11.53	—5.6
	27–31	5.8	3.3	0.13	8.2	11.63	—5.8
	29–32	6.3	3.1	0.13	8.2	11.43	—5.1
IV.....	39–42	39.2	11.9	5.3	7.3	24.5	+14.7
	42–45	42.2	16.6	5.7	7.6	29.9	+12.3
	36–45	40.0	12.7	4.0	7.7	24.4	+15.6
	51–56	6.5	4.7	0.11	7.9	12.7	—6.2
V.....	59–64	5.4	4.0	0.15	6.5	10.7	—5.3
	66–71	6.7	3.7	0.11	6.8	10.6	—3.9
	72–77	7.0	3.4	0.18	6.7	10.3	—3.3
		D.—INSOLUBLE PHOSPHORUS.					
I.....	1–6	22.1	10.2	.....	2.5	12.7	+9.4
II.....	11–14	13.5	13.5	.....	2.4	15.9	—2.4
	13–17	13.4	6.8	.....	2.3	9.1	+4.3
	16–19	13.4	6.7	.....	2.4	9.1	+4.3
III.....	25–29	6.7	5.0	.....	2.2	7.2	—0.5
	27–31	6.8	5.5	.....	2.3	7.8	—1.0
	29–32	7.1	4.8	.....	2.1	6.9	+0.2

TABLE IV.—INTAKE AND OUTGO OF NITROGEN AND PHOSPHORUS—*Continued.*

D.—INSOLUBLE PHOSPHORUS—*Continued.*

Period.	Days.	Intake.	Outgo.				Balance.
			Dung.	Urine.	Milk.	Total.	
		Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
IV.....	39–42	2.4	5.5	.....	1.9	7.4	—5.0
	42–45	3.9	6.8	.....	1.8	8.6	—4.7
	36–45	3.8	6.5	.....	2.0	8.5	—4.7
	51–56	7.1	5.4	.....	2.4	7.8	—0.7
V.....	59–64	5.6	3.8	.....	1.9	5.7	+0.1
	66–71	5.0	5.1	.....	2.0	7.1	—2.1
	72–77	6.0	5.6	.....	2.0	7.6	—1.6
		E.—SOLUBLE ORGANIC PHOSPHORUS.					
I.....	1–6	51.1	3.4	.....	.....	.....	+47.7
II.....	11–14	5.4	1.8	.....	.....	.....	+3.6
	13–17	5.4	0.2	.....	.....	.....	+5.2
	16–19	5.3	1.4	.....	.....	.....	+3.9
III.....	25–29	3.6	0.9	.....	.....	.....	+2.7
	27–31	3.4	0.9	.....	.....	.....	+2.5
	29–32	3.7	0.9	.....	.....	.....	+2.8
IV.....	39–42	36.2	3.2	.....	.....	.....	+33.0
	42–45	38.8	7.5	.....	.....	.....	+31.3
	36–45	36.6	4.8	.....	.....	.....	+31.8
	51–56	2.6	2.2	.....	.....	.....	+0.4
V.....	59–64	1.7	1.8	.....	.....	.....	+0.9
	66–71	2.8	1.5	.....	.....	.....	+1.3
	72–77	3.1	1.6	.....	.....	.....	+1.6
		F.—INORGANIC PHOSPHORUS.					
I.....	1–6	3.3	37.0	2.2	7.0	46.2	—42.9
II.....	11–14	5.3	5.8	0.30	8.2	14.3	—9.0
	13–17	5.2	5.3	0.18	8.5	14.0	—8.8
	16–19	5.2	4.6	0.17	8.4	13.2	—8.0
III.....	25–29	2.3	2.3	0.13	8.3	10.7	—8.4
	27–31	2.4	2.4	0.12	8.2	10.7	—8.3
	29–32	2.6	2.2	0.13	8.2	10.6	—8.0
IV.....	39–42	3.0	8.7	5.3	7.3	21.3	—18.3
	42–45	3.4	9.1	5.7	7.6	22.4	—19.0
	36–45	3.4	7.9	4.0	7.7	19.6	—16.2
	51–56	4.0	2.5	0.11	7.9	10.5	—6.5

TABLE IV.—INTAKE AND OUTGO OF NITROGEN AND PHOSPHORUS—*Concluded.*
F.—INORGANIC PHOSPHORUS—*Concluded.*

Period.	Days.	Intake.	Outgo.				Balance.
			Dung.	Urine.	Milk.	Total.	
		Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
V.....	59–64	3.7	2.2	0.15	6.5	8.8	—5.1
	66–71	3.9	2.2	0.11	6.8	9.1	—5.2
	72–77	3.9	1.8	0.18	6.7	8.7	—4.8

TABLE V.— DISTRIBUTION AND BALANCE OF CALCIUM, MAGNESIUM AND POTASSIUM.

	Days.	Intake.	Outgo in—				Balance.
			Milk.	Urine.	Dung.	Total.	
Calcium:		*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*	*Grams.*
I.....	1–6	29.5	13.9	0.8	12.4	27.1	+2.4
III.....	23–32	32.5	14.7	2.8	18.4	35.9	—3.4
IV.....	43–45	56.5	12.9	0.6	37.5	51.0	+5.5
V.....	74–77	34.3	13.6	3.3	30.5	47.4	—13.1
Magnesium:							
I.....	1–6	22.2	1.07	5.2	11.3	17.6	+4.6
III.....	23–32	8.7	1.17	4.7	10.1	16.0	—7.3
IV.....	43–45	11.6	1.10	2.0	12.9	16.0	—4.4
V.....	74–77	10.5	1.00	1.8	10.6	13.4	—2.9
Potassium:							
I.....	1–6	106.7	14.2	60.9	56.3	131.4	—24.5
III.....	23–32	46.9	13.4	35.9	26.3	75.6	—28.7
IV.....	43–45	44.4	12.2	42.1	33.2	87.5	—43.1
V.....	74–77	41.6	10.4	49.0	23.2	82.6	—41.0

DISCUSSION OF THE DATA.

Nitrogen.— Jordan, Hart and Patten[1] make the statement that there is no relation whatever between the excretion of phosphorus and nitrogen. Two papers have since appeared which are interesting in this connection. The one reports experiments on four rabbits by Le Clerc and Cook,[2] showing that the addition of inorganic phosphorus to a normal ration increases the nitrogen meta-

[1] Jordan, Hart and Patten, N. Y. Agrl. Expt. Sta. Tech. Bull. No. 1, p. 48.
[2] Le Clerc and Cook, *Jour. Biol. Chem.*, 2: 303, 1906.

bolism, but when the phosphorus is added to a phosphorus-poor diet, the reverse is true. The same results were given by soluble organic phosphorus. These authors state that the phosphorus of bran is very readily absorbed, but that the nitrogen, on the other hand, is utilized very poorly; and quote Girard and Lindet[1] as having arrived at the same conclusion in their work upon rabbits. This, if true, is interesting with respect to the nitrogen in that it shows a marked difference in the nutritive economy among the species of herbivorous animals, for it is the common experience of agricultural chemists that cattle utilize bran proteins very well.[2] In Table III it is shown that our animal assimilated over 60 per ct. of the nitrogenous constituents of the rations, 78 per ct. of which was derived from wheat, and almost half of this from the bran. The other paper relates to an extensive experiment upon rats by Gregersen,[3] in which his purpose was to demonstrate that inorganic phosphorus is synthesized into protein phosphorus by the animal organism. He states definitely that there is a parallelism between the phosphorus and nitrogen elimination.

Sécheret,[4] studying the therapeutic value of phytin, claims that this phosphorus compound stimulates protein metabolism and increases the nitrogen elimination, but this is in direct conflict with the observations of Rogosinski,[5] who also worked on man. This latter author found no relation whatsoever between the protein and phosphorus metabolism when phytin, lecithin, and disodium phosphate were fed.

The experiments of this Station do not show any very striking relationships between these two excreted products; there is, however, an apparent resemblance between the curve of nitrogen and total phosphorus intake and outgo, as is shown in Figure 1. As Le Clerc and Cook state, the addition of phosphorus to a phosphorus-poor ration is followed by a decrease of the nitrogen output, immediately followed by an increase when the addition of phytate phosphorus is discontinued. This parallelism is independent of the relations between the apparent digestibility of the nitrogen and phosphorus, as is shown in Table III. In drawing conclusions from the data of this experiment, it must be borne in mind that the animal was not in nitrogen equilibrium and the amount of rations voluntarily consumed by the cow varied considerably in the latter period of the experiment; however, if we review the former experiments of this series and construct curves for them, we get very much the same

[1] Girard and Lindet Froment et sa monture, 1903.

[2] Jordan and Hall, U. S. Dept. Agr., O. E. S. Bull. 77, p. 79.

[3] Gregersen, *Ztschr. Physiol. Chem.* **71**: 49. 1901.

[4] Sécheret; Thése de Paris, 1904, p. 131, from Maly's *Johrber. Tierchem.*, **34**: 729. 1904.

[5] Rogosinski *Anz. Akad. Wiss. Krakau*, B 1910, p. 260; from *Chem. Centrbl.* **31, II**: 1558. 1910.

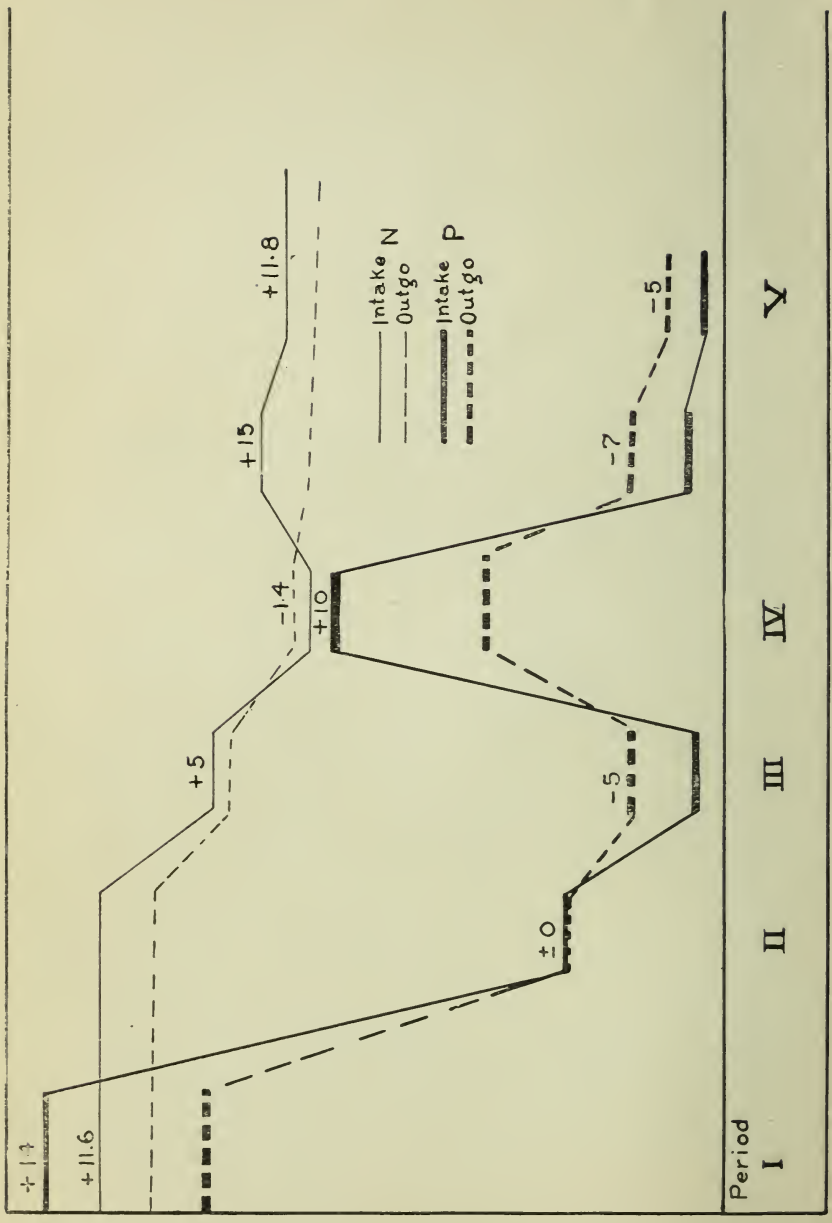

FIG. 1.—RELATION BETWEEN PHOSPHORUS AND NITROGEN METABOLISM.

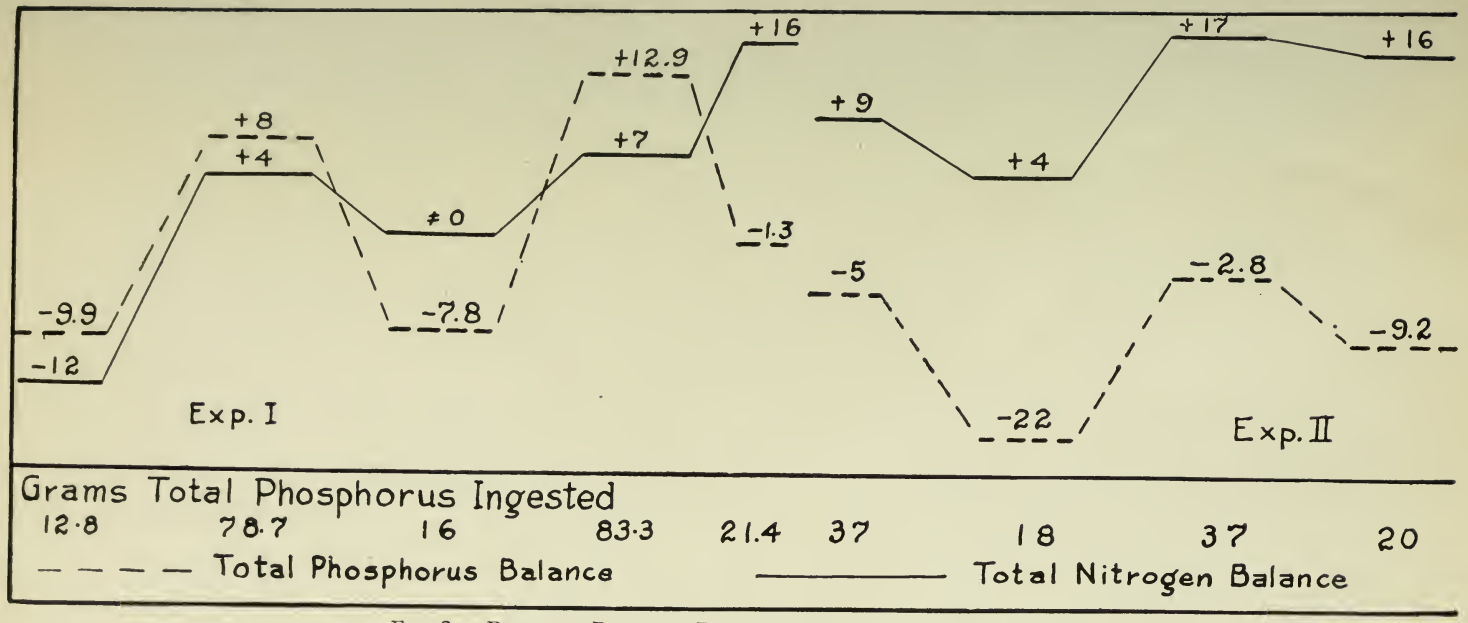

FIG. 2.—RELATION BETWEEN PHOSPHORUS AND NITROGEN BALANCE.
(Constructed from data in Technical Bulletin No. 1.)

picture (Fig. 2). In the first experiment, the intake of nitrogen was almost constant; the results of this experiment differ from those of the fourth experiment in that there exists a parallelism between the apparent digestibility of the nitrogen and the phosphorus intake. This is also true of Experiment Three. The second experiment shows no such parallelism, but this should not be given much weight in the argument inasmuch a new factor (nucleo-protein in the rations) is here involved which might be responsible for the discrepancy. In view of all this, one is almost justified in assuming that there may exist between phosphorus and nitrogen metabolism some intimate relationship involved in the synthesis or cleavage of the nucleoproteins in the organism, but the facts so far established are not sufficient to warrant any conclusion on the fundamental principles here suggested.

Total phosphorus.— The ingested phosphorus was mostly eliminated by way of the intestines. The amount of phosphorus excreted in the urine was relatively very small, as little as one-half of one per ct. of the total outgo of this element on a phosphorus-poor diet. The amount of phosphorus in the urine is readily changed by increasing the phosphorus intake (Period IV, Table IVb), as is evident from the study of the data of the phytin period, where the urine phosphorus rose to 15 per ct. of the total outgo. The response to the change in the ration was immediate. Twenty-five grams of phytin were withheld on the forty-sixth day, the next day's urinary phosphorus was 1.31 grams less than on the preceding day; on the fifty-first day, the phytin intake had been reduced to the minimum and the corresponding urine had 90 per ct. less of phosphorus. The same effects are also shown in the other transition periods. In the days between periods I and II there is an immediate drop of 50 per ct. in response to the substitution of washed bran for whole bran. These cases cited above are those of reduction of phosphorus in the rations. When it is a matter of increase the quantitative change is equally striking, but follows a lag of two days. One set of figures will illustrate this point: On the 36th day, there was 0.3 grams P in the urine; 37th day 0.24 grams; 38th day, 0.23 grams; 39th day, 3.85 grams; 40th day, 44.1 grams P in the urine, the full portion of phytin, 175 grams, having been added on the thirty-seventh day. These quantitative changes of the phosphorus in the urine were not wholly due to the relative total amounts of this element in the rations, but also to the nature of its chemical combination or the internal relations of the phosphorus in the feeds themselves. No other assumption can explain the differences between periods I and IV in which the urine of the former contains less phosphorus than that of the latter, though in its ration there was twice as much phosphorus, and in both periods more than half of the ingested phosphorus was in the form of phytin. In comparing the experiments II and III by Jordan, Hart and Patten,

we have a suggestion of the difference induced by the nature of the chemical combination of the phosphorus in the rations. In Experiment II, which is a study of nucleo-protein phosphorus, there was no change in the phosphorus content of the urine but in the other experiment, which was a phytin problem, there is recorded a drop of 99 per ct. when the soluble organic phosphorus compound is withdrawn. Experiment II should be repeated before definite conclusions are drawn, not only because it is a single experiment, but in that the amount of phosphorus fed in its principal period was too small to maintain even an equilibrium of this element.

The minimum amount of phosphorus in the urine did not occur in the samples from the end of the protracted low-phosphorus period, as one might have expected; the urine of this period was no poorer in phosphorus than that of Period III. The lowest urinary phosphorus occurred in the week following the last feeding of calcium phytate.

From Table IV we see that the animal maintained approximately a phosphorus equilibrium on an intake of twenty-four grams of total phosphorus while giving milk carrying eleven grams of the element, leaving thirteen grams per day for the other physiological functions. The phosphorus requirement aside from the milk production would therefore seem to be about twenty-six milligrams per kilo body weight, as a minimum for this animal. When less phosphorus is given than the organism requires, the physiological functions continue at the expense of the phosphorus previously stored in the tissues of the body. Such a storage takes place when a greater amount of phosphorus than is indicated above is being fed, but this is not in direct proportion to the amounts of the increase as can be readily seen by studying periods I and IV in Table IV. Twenty days of very low phosphorus intake did not materially change the phosphorus balance. This is in harmony with previous experiments.

Insoluble phosphorus.—The insoluble phosphorus was obtained, as in the previous work, by subtracting the soluble phosphorus from the total phosphorus, and was considered, as in the former experiments, to be chiefly nucleo-protein phosphorus. There is less insoluble phosphorus in the washed bran than in the original, because it is carried out mechanically by the water along with considerable starch and gluten.

An inspection of Table IV shows that when the amount of insoluble phosphorus in the ration is changed, the amount of this form of phosphorus in the feces also changes; but when the insoluble phosphorus in the feed is reduced to 13.5 grams per day, further reduction does not result in corresponding decrease in the amount of this form of phosphorus in the dung. The insoluble phosphorus was very largely changed to inorganic phosphorus. This is not apparent when the amount of intake was less than 13.5 grams, as is indicated by the data in the table for the days following number 13, in which

there seems to be an approximate regularity in the excretion of insoluble phosphorus independent of the amount of this form of phosphorus ingested.

Soluble organic phosphorus.—In the organic phosphorus compounds soluble in 0.2 per ct. hydrochloric acid, we have the phytin of the grains and feces and such small amounts of glycero-phosphates as may occur in the feces. The amount of soluble organic phosphorus in the dung is relatively very small, less than 5 per ct. of the total phosphorus passed from the animal body, except in the fifth period when the phytin content of the feces increased. Only a very small amount of phytin is excreted into the milk and urine.[1] It forms less than 3 per ct. of the urinary phosphorus and its presence in the milk has not yet been sufficiently demonstrated. In this experiment, the soluble organic phosphorus in the milk and urine was not deemed significant at the time the analyses were made and its determination therein were omitted. Our study of phytin phosphorus is therefore confined to the figures obtained from the analyses of the rations and feces. In Table III, where these data are recorded, we see a relatively small amount of phytin phosphorus in the feces, even in the first and fourth periods when the phytin intake was 51.1 grams and 36.6 grams per day respectively, from which we must conclude that the phytin disappears from the alimentary tract to a very large extent. It is readily absorbed and in the tissues is hydrolyzed by enzymes and converted into inorganic phosphate and inosite.[2] Any which fails of absorption, or is returned to the intestine after absorption, may also be split by intestinal bacteria, specifically *B. coli*.[3] Rogosinski, whose work was referred to on p. 15 found that human fecal matter completely destroyed phytin. The dog on the other hand eliminated 70 per ct. of the administered phytin without any change in it. The other 30 per ct. was assimilated and a corresponding amount of inorganic phosphorus eliminated in the urine. Hence considering the enormous bacterial flora of the cow's interior it is not surprising to find that only 6.5 per ct. of the soluble organic phosphorus fed in the first period was recovered in the feces. The apparent utilization of the phytin in the calcium phytate period was appreciably less, ranging from 89 to 91 per ct., with one-third more soluble phosphorus in the feces than in the first period, although less phytin was fed. In Table IV, a comparison between the total phosphorus ingested and the phosphorus balance in periods 3, 2 and 4, shows that the output fluctuates with the intake, hence there is a more direct relation between the two than is observed in nitrogen metabolism, in which the balance is not so immediately influenced by the amount ingested. The

[1] Starkenstein. *Biochem.* Ztschr. **30**: 56. 1910.
[2] McCallum and Hart, *Jour. Biol. Chem.* **4**: 497. 1908.
[3] Unpublished data by the author.

average intake of total phosphorus in the transition period between periods 1 and 2 is 51.2 grams, and the outgo is 46.3 grams, giving a balance less than any in the calcium phytate period with an average ingestion of 43.8 grams of total phosphorus per day. The apparent digestibility of phytin phosphorus in the low-phosphorus period is even less. These figures suggest that the various phytins are not utilized with equal ease, and those which were less readily washed out of the original bran were the more apt to pass through the alimentary tract unchanged. This is purely speculative; more data are required before an intelligent interpretation can be made.

Inorganic phosphorus.— In this experiment, as in the previous work, there was in all cases more inorganic phosphorus eliminated than had been given in the rations. The end product of phosphorus metabolism is inorganic phosphate, which in the herbivora is excreted chiefly by way of the intestinal canal as salts of the alkali earths.[1]

Bases.— In all the periods, more potassium was excreted than was taken into the system. The amount of this element in the bran was reduced by the leaching to which it had been subjected, so that there was, therefore, a lessened intake in the periods during which washed bran was fed, accompanied by a decreased elimination in the feces and urine. On the addition of calcium phytate, there was an increase of potassium in the dung and urine amounting to somewhat more than six grams in each; on the withdrawal of calcium phytate, fecal potassium fell ten grams per daily output but the urinary potassium slightly increased.

The whole-bran period gave a magnesium balance of + 4.6; all the other periods were deficient in this element. The magnesium differed from the calcium and potassium in that the amount in the urine decreased constantly from the first period to the end of the experiment. The decrease was most marked between the third and fourth periods, probably due to the influence of calcium phytate which seemed to draw the magnesium toward the intestinal canal. The fecal magnesium was quite constant, about ten grams per day, except in the fourth period when it seems to have been influenced by the increased calcium intake. At the beginning of the experiment, about half as much magnesium was excreted in the urine as in the feces, but at the end, when the labile magnesium of the body had been largely exhausted, only about one-fifth as much was eliminated in the urine.

The calcium elimination in the urine increased remarkably when the phosphorus intake was diminished, and fell again to its former level when the phosphorus was increased. In the last period, when the low phosphorus ration was given for a long time, the calcium in the urine rose to five times the amount excreted through this channel in the phytin period. The calcium in the dung also in-

[1] According to Berg as tri-basic calcium phosphate, *Biochem. Ztschr.* 30: 107. 1910.

TABLE VI.— AVERAGE STATEMENT OF THE DAILY YIELD AND COMPOSITION OF THE MILK, AND ITS CONTENT OF FAT, NITROGEN AND PHOSPHORUS.

Period.	Day Nos.	Milk.	Fat.	Solids.	Ash.	Total nitrogen.	Casein nitrogen.	Total phosphorus.	Fat.	Nitrogen.	Phosphorus.
		Kilos.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Grams.*	*Grams.*	*Grams.*
I Whole bran period...	1–6	9.29	3.75	12.9	0.805	0.63	0.47	0.101	346.1	58.7	9.5
II Phosphorus equilibrium period....	11–14	9.83	3.8	12.92	0.805	0.59	0.45	0.111	372.3	57.7	10.6
	13–17	9.55	3.76	12.95	0.797	0.60	0.46	0.112	362.3	57.8	10.8
	16–19	9.73	3.8	12.97	0.807	0.60	0.47	0.111	369.6	57.3	10.8
	11–19										
III Low phosphorus period.....	25–29	9.82	3.16	12.23	0.859	0.57	0.436	0.107	310.1	55.7	10.5
	27–31	9.91	3.20	12.14	0.865	0.57	0.436	0.107	307.7	56.0	10.5
	29–32	9.77	3.05	12.34	0.863	0.57	0.436	0.106	302.3	55.4	10.3
	23–32										
IV Calcium phytate period	36–40	9.15	3.46	12.26	0.854	0.58	0.430	0.109	314.9	53.2	10.4
	39–42	8.34	3.70	12.32	0.840	0.555	0.430	0.111	308.5	46.3	9.2
	42–45	8.83	3.50	12.58	0.859	0.555	0.440	0.109	308.6	48.5	9.4
	36–45	8.99	3.50	12.43	0.849	0.560	0.430	0.109	314.7	50.6	9.7
V Protracted low phosphorus period...	51–56	9.50	3.6	12.64	0.840	0.57	0.45	0.108	340.3	54.1	10.3
	59–64	8.33	3.6	12.25	0.825	0.55	0.43	0.102	290.4	45.3	8.4
	66–71	8.28	3.4	12.24	0.816	0.55	0.43	0.107	280.8	45.4	8.8
	72–77	8.44	3.25	12.22	0.833	0.54	0.43	0.103	277.8	45.6	8.7

creased with the decrease of phosphorus. The increase in the calcium of the feces in the calcium-phytate period was equivalent to about the amount of calcium increase in the ration. In the last period, the calcium was very much in excess of the calcium in the feces of the first two periods.

INFLUENCE OF PHOSPHORUS ON THE MILK.

The remarkable results of the work reported by Jordan, Hart and Patten in which it was clearly shown that the removal of various compounds from the bran influenced both milk fat and milk flow were confirmed in this experiment, though not in so striking a degree. The data in Table VI are graphically presented in Fig. 3 whose ordinates represent the percentage of fat in the milk, the amount of fat in the milk and the amount of milk flow. The abscissa is not used here for quantitative representation. The base line is divided into parts representing the successive periods, with space for the transition periods, and the amounts of phosphorus given are also indicated here by numerals. Each curve has a different scale and is placed at a convenient distance from the others to facilitate comparison. The top curve (A) represents the percentage of fat in the milk and is drawn to a scale of one-tenth of 1 per ct. to .03 inch; the middle curve (B) represents the total amount of fat and is drawn to a scale of 4 grams to .03 inch; the lowest curve (C) represents the milk yield and is drawn to a scale of fifty grams to .03 inch. For comparison, the results of the previous experiments (by Jordan, Hart and Patten), are plotted in a similar manner (Fig. No. 4). The curves have a general tendency to decline as the period of lactation progresses; this makes the high average yield during the week following the end of the calcium phytate period significant. In all cases where the phosphorus of the ration increases it is immediately followed by a drop in the milk flow, and the withdrawal of phosphorus is followed by a larger yield of milk. Between the week of rapid rise in milk flow which followed the calcium phytate period, and the prolonged low phosphorus period, there is a large decline due to some unknown factor, probably lack of appetite and associated disturbances, but during this low phosphorus period, the milk flow was on a gradual though small increase until the final break which caused the discontinuance of the experiment. These changes are small — from 2 to 20 per ct. in experiments I, II, and III, and from 5 to 7 per ct. in experiment IV — but the results are consistent, with the exception of the last period of experiment III, in which are probably shown the effects of protracted malnutrition. The line representing the fat content of the milk moves up and down regularly as the amount of phosphorus in the ration increases and decreases. The response is in this case also immediate. The change is not a mere matter of fluctuation in

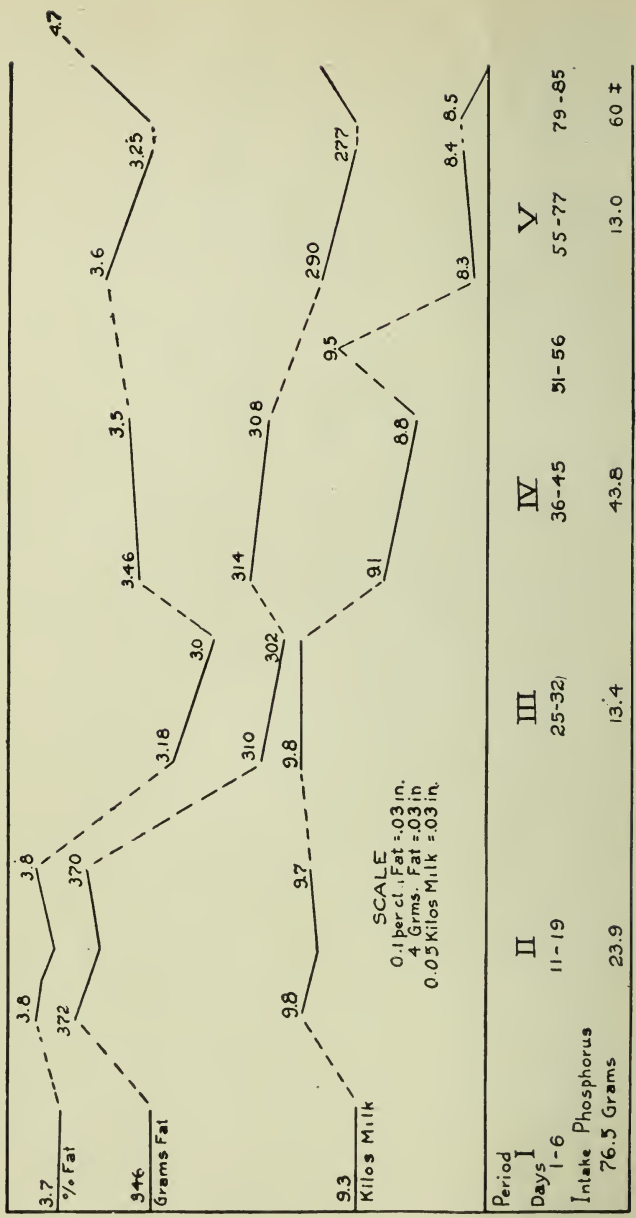

FIG. 3.—INFLUENCE OF PHOSPHORUS INTAKE ON PERCENTAGE AND AMOUNT OF FAT AND MILK FLOW.

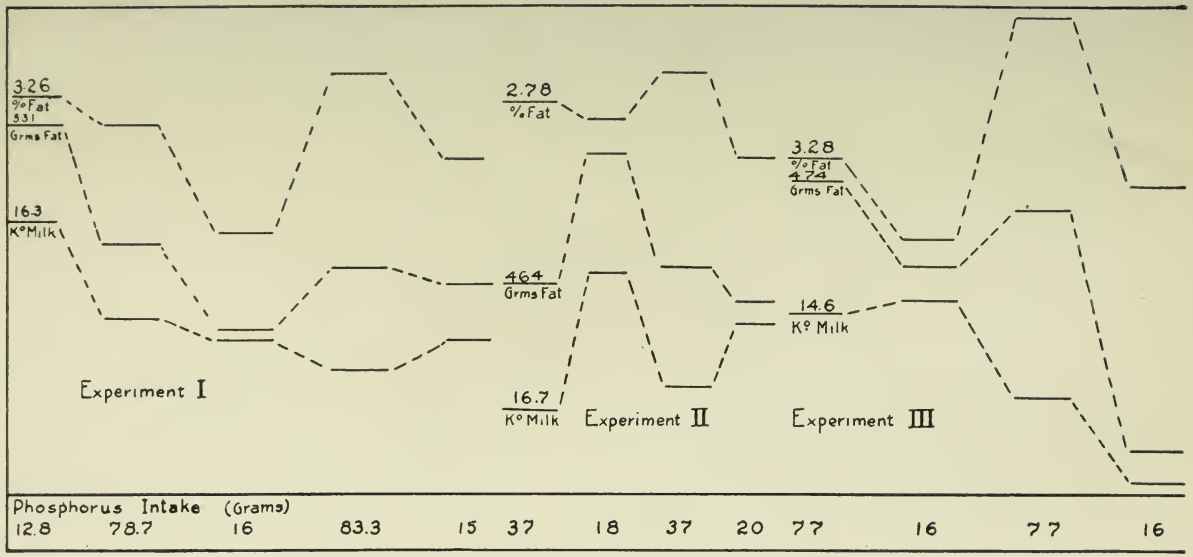

FIG. 4.—INFLUENCE OF PHOSPHORUS ON PERCENTAGE AND AMOUNT OF FAT AND MILK FLOW.
(Constructed from data in Technical Bulletin No. 1.)

water content, but an actual increase of fat secretion by the mammary glands, as is most strikingly shown by the middle line in period 4 of Fig. 3, recording an actual fat increase at the time of a milk decrease. The relative differences in the fat production in the several experiments, induced by the change in the rations were not constant. In experiment I this difference amounted to 24 per ct. of the total fat, while in experiments II and III, it was only 5 per cent; in experiment IV the differences in the fat output of the several periods were relatively greater than in the previous work, but the largest difference was less than the largest difference in experiment I.

The period of phosphorus equilibrium shows the most favorable fat and milk production, which in this particular instance amounts to seven ounces fat and two and one-half pounds of milk per week in excess of the production on the normal rations. This can readily be explained by the assumption that the change in the ration was sufficient to change the milk flow but not enough to lower the relative amount of fat in the milk. If further experiments establish this as a physiological fact it will be interesting scientifically and practically suggestive.

Aside from the fat, the tables show but a relatively small change in the composition of the milk. Since the fat has been shown to vary with the rations, it should be deducted from the total solids, before a study of the solids is undertaken. In Table VII, in which the average composition of the milk for each period is given, the fat-free solids and the fat-free, ash-free solids are listed in columns six and eight. The differences between the percentages are small for both the solids and the ash. The small variations seem to follow the changes in the milk flow, so that the actual increase in the milk solids resulting from the decrease in the phosphorus in the ration is somewhat more pronounced than the changes in the milk volume, the same being true of the decrease when calcium phytate was added. The increased milk flow may therefore be considered as a true secretion of milk and not a mere dilution analogous to polyuria.

Table VII.— Yield and Composition of the Milk in the Several Periods.

Day Nos.	Milk.	Fat.	Solids.	Fat-free solids.	Ash.	Fat-free, ash-free solids.	Total nitrogen.	Casein nitrogen.	P.	Ca.	K.	Mg.
	Kgs.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
I 1–6 ...	9.29	3.75	12.9	9.15	0.805	8.3	0.63	0.47	1.01	0.147	0.155	0.011
II16–19...	9.73	3.8	12.97	9.17	0.807	8.36	0.60	0.47	1.11	.....	.....	.....
III29–32...	9.77	3.05	12.34	9.29	0.863	8.44	0.57	0.44	1.06	0.144	0.130	0.011
IV36–45...	8.99	3.50	12.43	8.93	0.849	8.05	0.56	0.43	1.09	0.145	0.136	0.012
V51–56...	9.50	3.60	12.64	9.04	0.840	8.2	0.57	0.45	1.06	.....	.....	.....
V72–77...	8.44	3.25	12.22	8.97	0.833	8.2	0.54	0.43	1.03	0.159	0.124	0.013

The tendency of the ash is to follow the milk solids. The several components of the ash mentioned in the table may be considered as constant, with the exception of potassium, which seems to decrease slightly throughout the experiment. On the 79th day, whole bran was introduced into the ration, and according to the data in the previous periods, one would expect a decrease in the milk flow and milk solids. The volume of milk had been decreasing throughout the long preceding period and no marked change was noted in the milk flow which could be ascribed to the whole bran; the solids, however, increased on the 82d day. This is obviously a different matter from the former fluctuations in the milk solids, and may be likened to the phenomena commonly observed at the end of a period of lactation. The analytical data for this period are as follows:

Number of day.....	77	78	79	80	81	82	83	84
Percentage of fat.....	3.25	3.35	3.25	3.2	3.6	3.8	4.4	4.7
Percentage of fat-free solids.	9.0	8.80	9.10	8.9	8.8	9.0	9.2	9.5

The percentages of total and casein nitrogen show very small but gradually decreasing values throughout the five periods, which is in contrast to the three former experiments of this series in which a small but gradual increase is indicated. These authors stated that aside from the change in the fat content there was no influence

TABLE VIII.—ANALYSIS OF MILK FOLLOWING PERIOD V; RATIONS THE SAME AS IN PERIOD I.

DAY.	Fat.	Solids.	Ash.	Total N.	Casein N.
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
78.....	3.35	12.12	0 810	0.55	0.43
79.....	3.15	12.26	0.863	0.55	0.44
80.....	3.20	12.15	0.840	0.56	0.44
81.....	3.6	12.37	0.803	0.57	0.44
82.....	3.8	12.88	0.848	0.55	0.45
83.....	4.4	13.67	0.777	0.57	0.44
84.....	4.7	13.24	0.825	0.55	0.44

TABLE IX.—AVERAGE PER DAY OF THE ASH CONSTITUENTS IN THE MILK.

Period.	K.	Ca.	Mg.	K.	Ca.	Mg.
	Per ct.	*Per ct.*	*Per ct.*	*Grams.*	*Grams.*	*Grams.*
I	0.155	0.147	0.011	14.19	13.88	1.07
III.....	0.130	0.144	0.012	13.34	14.07	1.19
IV.....	0.136	0.145	0.013	12.20	12.91	1.11
V	0.124	0.159	0 012	10.41	13.61	1.02

on the milk due to changes of the amount of phosphorus in the ration. Their tables justify such a conclusion; for in only one instance is there such a relation, namely in the fifth period of experiment I, where the following quantities are recorded:

Phosphorus fed.....	83.3	21.4	80.7	grams
Fat-free solids.....	8.3	8.6	8.2	per cent

If a change is induced in the composition of the milk by the variation of the phosphorus of the diet, we would expect this to affect the casein, but in all four experiments the casein varies but little and in no definite relation to the phosphorus intake. The other organic phosphorus compound of the milk, lecithin, was not determined. If we return to the total phosphorus and recalculate these amounts to a fat-free milk, we obtain the following percentages:

Period	I	II	III	IV	V
Phosphorus.....	0.107	0.115	0.108	0.113	0.106

This makes a maximum difference of 0.009 per ct. Similar calculation of the data from the experiments reported in Technical Bulletin No. 1, gives:

Experiment	I					II				III	
Grams P in rations	12 8	78.7	16.0	83.3	21.4	37.0	18.0	37.0	20.0	77.0	16.0
Percentage of P in fat-free milk....	0.090	0.089	0.086	0.083	0.090	0.108	0.108	0.107	0.110	0.104	0.110

or a maximum difference for the two animals of only 0.032 per ct. and for the one animal, "Nancy," used in experiments II, III and IV of only 0.011 per ct. which may well be considered within the limits of unavoidable error. This suggests that the phosphorus contents of the solids vary with the individual animal and not with the phosphorus content of the rations fed.

Until more evidence to the contrary has been brought forth we may safely assume that the fat is the only milk constituent changed by alteration of the phosphorus content of the rations, and that there is a definite relation between the phosphorus supply and the yield of milk and butter fat.

It has not been conclusively proven that all the changes noted in the milk production are due *specifically* to *phytin phosphorus* but the evidence warrants the assumption that this substance is in whole or in part responsible for the phenomena observed. When further experiments which have been planned for this series are completed it is hoped that the evidence will show more definitely just what part the phytin salts as a whole, or its cations and anion will play in the physiological functions of the milk cow and how the associated bran extractives may modify this action.

INFLUENCE OF PHOSPHORUS ON EXCRETA

There were in this experiment no gross changes in the nature of the dung like those reported in the previous experiment but changes

in the weight of the samples dried at 60° C. indicate different moisture-content of the feces of the several periods. This bears no relation to the consumption of water nor to the total phosphorus of the ration as is shown by the following figures:

Period	I	II	III	IV	V
Water intake, kilos.....	42.3	44.3	40.0	42.0	42.1
Phosphorus intake, grams...	76.5	24.0	13.4	43.8	13
Water in feces, kilos.....	20.0	18.0	17.6	15.7	20.0
Water in feces, per ct.....	86.0	81.5	83.5	79.3	82.8

From experiences in past work in this Station, one would expect dryer feces to result from the change from a whole-bran ration to one in which the bran was washed. This we see to be the case if we examine the eighth column of Table X. The moisture of the feces drops gradually, in a four-day lag, from 86 per ct. in period I to 81.5 per ct. in period II. With a further decrease of the phosphorus in the ration, the moisture of the feces rose instead of

Table X.— Intake and Outgo of Water.

Period.	Days.	Water.					Per-centage in dung.	Difference between intake and outgo.	Atmos-pheric temp.*
		Intake.	Outgo in—			Total outgo re-corded.			
			Milk.	Urine.	Dung.				
		Kgs.	*Kgs.*	*Kgs.*	*Kgs.*	*Kgs.*	*Per ct.*	*Kgs.*	° F.
I	1-6	42.3	8.28	9.39	20.1	37.5	86	4.8	62.5°
II	13-17	44.2	8.34	8.09	17.4	33.8	81.7	10.4	79°
	16-19	44.5	8.46	7.36	18.6	34.4	81.4	10.1	78°
III	27-31	39.7	8.61	7.98	17.8	33.8	83.5	5.9	62°
	29-32	40.2	8.64	7.62	17.4	33.6	83.4	6.6	61°
IV	42-45	42.7	7.72	7.52	15.4	30.6	79.1	12.1	70°
	36-45	41.2	7.83	7.30	16.0	31.1	79.5	10.1	68°
V	66-71	42.2	7.15	6.22	19.7	33.0	82.9	9.2	81°
	72-77	42.1	7.35	6.88	20.0	34.5	82.6	7.6	80°

* The temperature recorded was kindly furnished by Mr. Newell and is the average maximum per day, taken in a shaded and properly ventilated place about 150 feet from the metabolism room.

becoming less, though the animal took in 4.3 kilos less of water per day. The laxative effect observed in previous experiments was probably due to some other factors than the phosphorus compounds or potassium and magnesium. The ingestion of phosphorus, potas-

sium and magnesium was about the same in the fifth period as in the third; a little more water was consumed but the feces were somewhat drier. If the laxative effect of wheat bran is due to the phytin anion, one would expect a wetter feces in the fourth period when 175 grams of calcium phytate was administered with the rations, but the dung of this period contained less water than that of any other. It is interesting to note however that the average moisture content of the dung of the second four days after the beginning of the phytate feeding was 87.6 per ct. but it soon dropped and gave an average for the period of less than eighty per ct. It is a peculiar incident that the cow desired more water when the moisture in each hundred grams of feces became less. The intake of water seems not to influence the amount of this substance passed out through the intestine.

It has been noted above that the volume of the milk changed in an inverse order to the amount of phosphorus intake, as may be seen from the table of water intake and outgo. (Table X.) The volume of urine shows no parallelism either with the amounts of phosphorus or of water ingested and therefore differs from previous experience in this series of problems. It is probably another peculiar coincidence that the water intake and water outgo in the urine if plotted give curves which run in opposite directions to one another. There is however a suggestion that the urine fluctuates inversely with the temperature of the atmosphere, which is probably accounted for by differences in the amount of perspiration. A curve plotted from the figures obtained by subtracting the values for the water content of dung, urine and milk from those for the water content of the ration (column 9, Table VIII) resembles a similar curve plotted for the atmospheric temperature during the time of the experiment. In the fourth and fifth periods the differences between the temperature and the water balance are such as to lead one to suspect a marked retention of water. The moisture elations in the problem are probably significant, but under the circumstances, inasmuch as a calorimeter was not available, actual determinations could not be made, and a true water balance is therefore impossible.

GENERAL PHYSIOLOGICAL CONDITION.

At the beginning of the experiment, the animal was in good health, and the rations were so planned that she should have an abundance both of protein and carbohydrates. During the experiment, the cow gained 19 kilos in body weight. At first she ate well, cleaning up all the rations offered, but beginning with the 17th day, ten days after washed bran had first been administered in place of the whole bran, she refused varying amounts of feed. The introduction of small amounts of aromatic substances did not seem to

improve the ration from the cow's point of view. At first the amounts refused were small and irregular, but as the experiment progressed, they became increasingly larger and occurred every day, so that it was deemed advisable to decrease the rations in the fourth period. The lack of appetite was at first considered as a natural aversion on the part of the animal to the ration. But as she ate the ration during the first week with relish such aversion must then have developed from monotony. It is more probable, however, that there was no real dislike for the food but a decreased appetite due to physiological disturbances. Certain remarks of Professor Mendel in a recent public lecture suggested this idea. These were based on his observations on experiments with rats and the data reported by Hart and McCollum. In Science for November 24, 1911, the following sentences occur in Mendel and Osborne's article: "And whereas nutritive decline has commonly been attributed to the anorexia consequent upon the monotony of diet, we are more than ever inclined to shift the explanation in many such cases to malnutrition as a primary cause. From this point of view improper diet and malnutrition may be the occasion rather than the outcome of the failure to eat — a distinction perhaps not sufficiently recognized heretofore." The interesting and significant data obtained by Mendel and Osborne are published in Carnegie Institution Publications No. 156. Hart & McCollum's data are published as Research Bulletin No. 17 of the Wisconsin Agricultural Experiment Station. The addition of calcium phytate did not mitigate the situation. Up to the fifth period, the animal showed no outward signs of constitutional disturbance except this constant decrease of appetite but by the close of this period untoward symptoms resulted from the protracted malnutrition. It is interesting to note that the weight of the cow increased eleven kilos during the last period, which, judged by her appetite, was the least promising period in the whole experiment. The increase in weight during the first two periods was almost at the rate of one kilo per day; for the next thirty-four days, i. e., to the end of the fourth period, the weight remained approximately constant, in the last period, the average weight was nine kilos more than in the two preceding periods. Of the total increase in weight during the whole experiment, less than half can be accounted for by the nitrogen balance. From the eightieth day, the animal began to develop pathological conditions, difficult to describe, but perfectly apparent to the men on the farm familiar with live stock. She seemed in good flesh and showed a glossy coat but refused to eat, manifesting positive distaste for her food. Her limbs became stiff and somewhat enlarged about the joints, and her movements were decidedly awkward. She was prone to lie down and had difficulty in getting upon her feet. There was no disturbance of the oestrum throughout the

experiment. The milk flow, which had begun to decrease on the seventy-seventh day, dropped rapidly to four kilos. As it was not permissible to slaughter the animal nor cause her permanent injury, the experiment was closed on the eighty-sixth day, and the cow removed to a box stall where she was given a plentiful supply of alfalfa and silage and whole bran. In the course of a week there were no more signs of malnutrition; she maintained the weight she had gained, the stiffness passed off, and the milk flow increased according to the following records kindly furnished by Mr. Smith, Station Dairy Expert.

Date	Number of days	Amount of milk
July 23	86	3.76 kilos
25	88	3.90
26	89	4.89
27	90	5.62
28	91	5.99
29	92	6.67

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New York Agricultural Experiment Station.

GENEVA, N. Y.

PHYTIN AND PYROPHOSPHORIC ACID ESTERS OF INOSITE. II

R. J. ANDERSON

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*Riverhead, N. Y. †Absent on leave. ‡Connected with the Chautauqua Grape Work.

TECHNICAL BULLETIN No. 21.

PHYTIN AND PYROPHOSPHORIC ACID ESTERS OF INOSITE. II.

R. J. ANDERSON.

SUMMARY.

Several new salts of phytic acid are described, viz.: The calcium-magnesium-potassium phytate, the penta-calcium phytate, the tetra-calcium phytate, the penta-magnesium phytate, the copper salts obtained when precipitating phytic acid with copper acetate, the octa-silver phytate and the hepta-silver phytate.

Efforts to synthesize phytic acid by acting on dry inosite with dry pyrophosphoric acid lead to the formation of esters.

Two of these, viz., the di-pyrophosphoric acid ester of inosite and a di-inosite tri-pyrophosphoric acid ester were obtained in pure form and analyzed.

These esters are very similar to phytic acid in appearance, taste and reactions. They yield similar acid salts and on hydrolysis inosite and phosphoric acid are produced.

INTRODUCTION.

In the last report[1] from this laboratory on the chemistry of phytin, various salts of phytic acid were described, as well as the tetra-phosphoric acid ester of inosite. Since then the investigation has been continued in connection with another problem dealing with the form in which phytin exists in wheat bran, which is not yet finished, but as the present work is closely related to that reported earlier, it seems advisable to publish it at this time.

In addition to the salts of phytic acid described before, the following have been prepared:

The calcium-magnesium-potassium phytate, $C_6H_{12}O_{27}P_6Ca_3Mg_2K_2$, a white amorphous powder obtained by neutralizing a solution of calcium-magnesium phytate in dilute hydrochloric acid with potassium hydroxide.

The penta-calcium phytate, $C_6H_{14}O_{27}P_6Ca_5$, is obtained as a white powder on precipitating an aqueous solution of phytic acid with calcium acetate.

The tetra-calcium phytate, $C_6H_{16}O_{27}P_6Ca_4 + 12H_2O$ is obtained as a white, semi-crystalline or fine granular powder when the above penta-calcium phytate in dilute hydrochloric acid solution is evaporated in vacuum in the presence of calcium acetate.

The penta-magnesium phytate, $C_6H_{14}O_{27}P_6Mg_5 + 24H_2O$, is obtained as crystalline powder when an aqueous solution of phytic acid and excess of magnesium acetate is evaporated in vacuum.

A copper salt corresponding to a hexacupric phytate, $C_6H_{12}O_{27}P_6Cu_6$, is obtained when phytic acid is precipitated with copper acetate.

[1] *Jour. Biological Chem.* 11: 471, (1912); and Tech. Bul. No. 19 of this Station.

The octa-silver phytate, $C_6H_{16}O_{27}P_6Ag_8$, is precipitated as a white amorphous powder by alcohol from an aqueous solution of phytic acid containing twelve equivalents of silver nitrate.

The hepta-silver phytate, $C_6H_{17}O_{27}P_6Ag_7$, results when the dilute nitric-acid solution of the above octa-silver phytate is precipitated with alcohol.

Since various attempts to synthesize phytic acid or to prepare a hexa-phosphoric acid ester of inosite by acting on inosite with phosphoric acid only lead to the formation of the tetra-phosphoric acid ester of inosite [1] it seemed of interest to determine what products would be formed when acting on inosite with pyrophosphoric acid. If phytin were a complex pyrophosphoric acid compound of inosite as suggested by Starkenstein [2] it appeared not impossible to synthesize it from these constituents. Such a synthesis would be of considerable theoretical and scientific value in connection with the chemistry of phytin and would also furnish an additional proof of the presence of pyrophosphoric acid compounds in nature.

Several futile efforts were made in this direction but it was found that the reactions tried only lead to pyrophosphoric acid esters of inosite. These esters are very easily formed but their purification is very difficult.

When acting on dry inosite (1 mol.) with dry pyrophosphoric acid (3 mol. or sufficient to form phytic acid) at a temperature of 200°–220° a new and stable ester is formed. On analysis, results were obtained corresponding to a di-pyrophosphoric acid ester of inosite, a compound isomeric with the tetra-phosphoric acid ester described in a former paper.

Attempts to isolate the reaction product by the method described for the tetra-phosphoric ester, [3] that is, by precipitating as a barium salt with alcohol, in the presence of hydrochloric acid, failed at first because barium pyrophosphate is equally insoluble in acidified dilute alcohol as barium phytate, for instance, or the pyrophosphoric acid esters. Various other salts were tried with negative results; the pyrophosphate invariably would be precipitated at the same time.

As is well known, pyrophosphoric acid when boiled with dilute mineral acids is very easily transformed into orthophosphoric acid. The isolation of the new ester was made possible by taking advantage of this property.

In the last paper [4] it was reported that phytin, when dry and free from mineral acids, is stable; that drying at 115° C. caused no appreciable decomposition and that no inosite could be isolated from 100 gms. of phytin after drying to constant weight at this temperature.

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[1] Anderson, *loc. cit.*

[2] *Biochem. Ztschr.* 30: 56.]

[3] Anderson, *loc. cit.*

[4] Ibid, *loc. cit.*

Experience since then has shown that phytin may be boiled for hours in dilute hydrochloric or sulphuric acid without suffering marked decomposition. In fact it may be boiled for days with 30 per ct. sulphuric acid without a determinable quantity of inosite being formed. This seemed strange as various other investigators have emphasized the fact that phytin is very easily hydrolyzed and that even in water it suffers a more or less rapid decomposition.

The action of nitric acid seems to cause a more rapid decomposition; for even the purest phytin when *warmed* in dilute nitric acid solution with ammonium molybdate gives very quickly the characteristic yellow precipitate of ammonium-phosphomolybdate. Several days, however, are required to cause complete decomposition in dilute nitric acid solution at a temperature of 60°–70° C. Quantitative experiments to measure the rate of decomposition have not been carried out, but it could very easily be done as the change is very slow.

The following will illustrate this point:

In an analysis of two different phytin preparations the substance was boiled with concentrated nitric acid under occasional additions of concentrated hydrochloric acid for about half an hour. At the end of this time the organic matter was apparently destroyed, as the solution was practically colorless. The phosphorus was determined in this solution by the usual molybdate method. After keeping at a temperature of 60° C. for one hour the precipitate was filtered off and the filtrate again warmed on the water-bath for another hour. A new portion of the yellow precipitate had then formed which was removed by filtration and the filtrate again warmed on the water-bath. A yellow precipitate continued to form slowly but continuously for two days when the experiment was discontinued. During this time the water lost by evaporation was replaced from time to time and small quantities of nitric acid were also added. The phosphorus determined in the first precipitate and in that which formed during the first day amounted to only 9.92 and 10.25 per ct., whereas when determined after first destroying the organic matter by the Neumann method 14.42 and 15.23 per ct. respectively were found.

In another case 100 gm. of calcium phytate was boiled under a reflux condenser with about 300 cc. of 30 per ct. sulphuric acid continuously for one day; over night it was heated on the water-bath and the next day the boiling was continued all day. After precipitating with excess of barium hydroxide, thorough washing in hot water, removal of excess of barium by carbon dioxide and evaporating on the water-bath, no inosite could be found in the slight residue which remained.

To determine if the phytin molecule suffered any partial decomposition on boiling with dilute acids, 1 gm. of phytic acid, dissolved

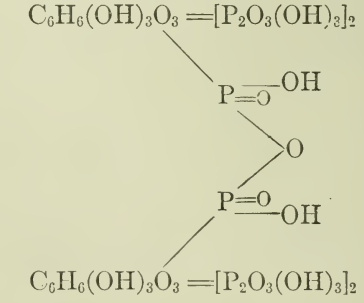
in 100 cc. of water acidified with 10 cc. 5/N hydrochloric acid, was boiled over a free flame for one hour. After cooling, barium chloride was added and the barium phytate precipitated by the addition of alcohol. The substance was twice purified by precipitating its hydrochloric acid solution with alcohol. On analysis, results were obtained which showed that the substance was a pure tri-barium phytate, the salt which is always obtained under the above conditions of precipitation.

In view of this relative stability of the phytin molecule it was thought that the pyrophosphoric acid ester referred to above might be more stable than the pyrophosphoric acid in the reaction mixture. Qualitative experiments showed that this was actually the case.

An aqueous solution of pyrophosphoric acid, acidified with hydrochloric acid and a solution of the above inosite-pyrophosphoric acid reaction-mixture, also acidified with hydrochloric acid, were boiled for one hour. Some barium chloride and a like volume of alcohol were then added. The solution containing only pyrophosphoric acid gave no precipitate, while, before boiling, alcohol produced at once a white precipitate of barium pyrophosphate. The solution containing the inosite-pyrophosphoric acid reaction mixture gave a white flocculent precipitate, the barium salt of the new ester.

As the excess of the pyrophosphoric acid was present as orthophosphoric acid after boiling it did not interfere with the purification of the compound by the dilute acid alcohol method.

By acting on dry inosite (one mol.) with dry pyrophosphoric acid (six mol.) at a temperature of 200°–220° C., another new pyrophosphoric ester was obtained. After boiling, as before, with dilute hydrochloric acid and purifying as the barium salt this product was found to be a di-inosite tri-pyrophosphoric acid ester; that is, its molecule was evidently made up of two molecules of dipyrophosphoric acid esters of inosite joined through one molecule of pyrophosphoric acid and, accordingly, it corresponds with the following formula:



It is evident therefore that complex compounds such as phytic acid is supposed to be cannot be formed at elevated temperatures,

as in the various reactions tried in these experiments only esters were produced, and at lower temperatures apparently no reaction takes place. These compounds are in physical and chemical properties very similar to phytic acid. They form analogous acid salts which in appearance and solubility seem almost identical with salts of phytic acid. Whether esters, such as above, are found in nature is at present unknown. It is, however, not impossible that a part of the organically bound phosphorus existing in plants may be present in some such, or similar, forms.

The silver salts previously referred to were prepared in the hope that they might serve for the preparation of an ester of phytic acid with which molecular weight determinations might be made. As was to be expected, however, only acid salts were obtained and, as such, were quite useless for the purpose in view. In the reaction between phytic acid and silver nitrate, nitric acid is of course liberated and when any strong acid is present only acid phytates are obtained.

Efforts made to prepare an ester by acting on sodium phytate with methylsulphate proved useless as no ester could be isolated. Further experiments along this line are contemplated and will be reported later.

In an article concerning the phosphorus compounds found in food materials which appeared in a Swedish chemical journal little known in this country and which is not abstracted by any of the larger chemical journals, a valuable contribution to the chemistry of phytin was made by A. Rising.[1] Among other things he describes a silver phytate of the following composition: C 5.5, H 1.08, P 13.2 and Ag 52.65 per ct., from which results he concludes that it must represent a complex pyrophosphoric acid compound of inosite. It is noteworthy that this author and E. Starkenstein,[2] independently, and practically at the same time expressed the same opinion, viz: that phytin represents a complex pyrophosphoric acid compound of inosite.

The silver salt described by Rising corresponds to the hepta-silver phytate mentioned in this paper. He proposes the following empirical formula: $C_6H_{14}Ag_3P_5O_{22}$, but his results agree equally well with a hepta-silver phytate: $C_6H_{17}O_{27}P_6Ag_7$.

	Calculated	Found by Rising	Found for hepta-silver phytate in this laboratory
C	4.92.....	5.50	
H	1.16.....	1.08	
P	12.72.....	13.20	13.02 per cent.
Ag	51.64.....	52.65	52.43 per cent.

From the above there appears to be no doubt that these salts are identical.

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[1] *Svensk Kemisk Tidskrift* 22: 143 (1910).

Note.— I am indebted to Mr. A. R. Rose of Columbia University for this as well as for many other valuable references to literature.

[2] *Loc. cit.*

The several salts of phytic acid reported in this paper were prepared from previously purified and analyzed phytic acid and for this reason it was deemed quite superfluous to make carbon and hydrogen determinations on each salt.

EXPERIMENTAL.

CALCIUM MAGNESIUM POTASSIUM PHYTATE.

Two gms. phytic acid was dissolved in about 100 cc. of water, 0.224 gm. of MgO (2 mol.), and 0.84 gm. $CaCO_3$ (3 mol.) added. The MgO dissolved at once and nearly all the calcium carbonate but the salt of phytic acid was precipitated at the same time as a white precipitate. This was dissolved by a few drops of hydrochloric acid, the solution filtered and the filtrate rendered slightly alkaline to litmus with potassium hydroxide. After the precipitate has settled it was filtered off, washed well in 50 per ct. alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a fine white amorphous powder. It was free from chlorine. On moist litmus paper it showed a faintly alkaline reaction. It was slightly soluble in water but readily soluble in dilute acids. Yield 2.9 gm.

After drying at 105° in vacuum over phosphorus pentoxide it was analyzed.

For $C_6H_{12}O_{27}P_6\ Ca_3Mg_2\ K_2 = 948$

Calculated Ca 12.65; Mg 5.12, K 8.24, P 19.60 per ct.

Found Ca 13.03; Mg 4.29, K 6.42, P 19.07 per ct.

This shows the difficulty of obtaining pure salts of phytic acid when several bases are combined in the same molecule of the salt.

PENTA-CALCIUM PHYTATE.

One gm. phytic acid was dissolved in about 50 cc. of water and excess of calcium acetate added. On the first addition of the calcium acetate a white precipitate is produced, but on shaking this redissolves and only after a liberal excess of the acetate has been added is the precipitate permanent. After settling, the product was filtered and thoroughly washed in 50 per ct. alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The substance was a perfectly white amorphous powder. On moist litmus paper it showed an acid reaction. It is only slightly soluble in water, readily soluble in dilute mineral acids, less soluble in acetic acid.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

For $C_6H_{14}O_{27}P_6Ca_5 = 904$

Calculated Ca 22.12: P 20.57 per ct.

Found Ca 22.46: P 20.62 per ct.

TETRA-CALCIUM PHYTATE.

Various attempts were made to obtain a penta-calcium phytate in crystalline form without success. A tetra-calcium phytate was finally obtained by the following method:

The penta-calcium phytate was dissolved in a small quantity of 0.5 per ct. hydrochloric acid, a concentrated solution of calcium acetate was added until a permanent precipitate remained which was then dissolved by the addition of a few drops of dilute hydrochloric acid. On now concentrating in vacuum to somewhat less than half the bulk at a temperature of 40° the calcium salt separates. The product was filtered off, washed thoroughly in 50 per ct. alcohol, alcohol and ether and dried in the air. The substance was a white semicrystalline or fine granular powder of irregular form. Its solubility was practically the same as for the penta-calcium phytate. It was free from chlorine.

On drying at 105° in vacuum over phosphorus pentoxide the substance lost water corresponding to 12 H_2O.

0.2120 gm. subst.: 0.0422 gm. H_2O.
0.1238 gm. subst. gave 0.0308 gm. CaO and 0.0967 gm. $Mg_2P_2O_7$.
0.1857 gm. subst. gave 0.0456 gm. CaO and 0.1448 gm. $Mg_2P_2O_7$.

For $C_6H_{16}O_{27}P_6Ca_4 = 866$
Calculated Ca 18.47: P 21.47 per ct.
Found Ca 17.78: P 21.77 per ct.
Ca 17.55: P 21.73 per ct.

For 12 H_2O calculated 19.96: found 19.90 per ct.

PENTA-MAGNESIUM PHYTATE.

After dissolving 2.5 gm. phytic acid in about 100 cc. of water, a concentrated solution of magnesium acetate was added. This did not cause any precipitate nor could the substance be brought to crystallization by any of the usual methods. The solution was then concentrated to about half its bulk in vacuum at a temperature of 35°–40°. As the concentration proceeded the substance began to separate as a heavy powder. This was filtered off, well washed in dilute alcohol, alcohol and ether and dried in the air. The product was a perfectly white semi-crystalline or loose granular powder of irregular form. On moist litmus paper it showed an acid reaction. It was slightly soluble in water, readily soluble in acids.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide. It lost water corresponding to 24 H_2O.

0.1504 gm. subst. gave 0.0510 gm. H_2O.
0.0997 gm. subst. gave 0.0671 gm. $Mg_2P_2O_7$ for Mg.
0.0498 gm. subst. gave 0.0393 gm. $Mg_2P_2O_7$ for P.

For $C_6H_{14}O_{27}P_6Mg_5 = 825.5$
Calculated Mg 14.71: P 22.53 per ct.
Found Mg 14.69: P 21.99 per ct.
For 24 H_2O Calculated 34.36: found 33.91 per cent.

HEXA–CUPRIC PHYTATE.

This salt is precipitated directly from phytic acid solutions by copper acetate. It is difficult, however, to obtain a pure compound as it is apt to contain either too little or too much copper, depending upon the conditions under which the precipitate is formed. In the purification of phytic acid it is usual to remove other bases which are present by repeatedly precipitating with barium chloride; the barium salt which is finally obtained is then decomposed with sulphuric acid. It is found, however, that if only the calculated quantity of sulphuric acid is used the barium sulphate which is formed is in an extremely fine condition which it is impossible to remove completely either by repeated filtrations or even by day-long centrifuging. But if a slight excess of sulphuric acid is used the barium sulphate in the course of only a few hours becomes heavy and granular and may be easily removed by simple filtration. In order to get rid of the excess of sulphuric acid the solution is now precipitated with copper acetate. The copper salt can be easily washed free of the sulphate and acetate with water as it is very slightly soluble in very dilute acids. The pure copper salt is then easily decomposed with hydrogen sulphide and the free phytic acid obtained.

The copper phytate obtained from such slightly acid solutions was analyzed and the following results obtained:

For $C_6H_{12}O_{27}P_6Cu_6 = 1083$
Calculated Cu 35.18: P 17.17 per ct
Found Cu 33.54: P 16.88 per ct.

Pure phytic acid in water was precipitated with pure copper acetate when a compound was obtained which had the following composition:

Cu 37.57: P 15.13 per ct.

It is seen from above that from slightly acid solutions of phytic acid a copper salt is precipitated which contains somewhat too little copper while from an aqueous phytic acid solution a salt is obtained which contains over 2 per ct. excess of copper.

The copper phytate is, like all other phytates, exceedingly soluble in 10 per ct. phytic acid. It dissolves readily until a thick heavy syrup is formed, but it was found impossible to bring this solution to crystallization. Both of the above copper salts show an acid

reaction on moist litmus paper. The red color is only developed slowly and is probably due to hydrolysis.

OCTA-SILVER PHYTATE.

This salt is obtained when an equeous solution of phytic acid, mixed with twelve equivalents of silver nitrate, is precipitated with alcohol. The product is a heavy, white, flocculent precipitate which settles at once. It was filtered off, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product is only slightly affected by light but on continued exposure turns first yellowish and later dark in color. In the dry state it is a heavy white amorphous powder of acid reaction on moist litmus paper. It is very soluble in dilute nitric acid and exceedingly soluble in phytic acid. Many attempts were made to bring it to crystallization from the latter solution without success.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

For $C_6H_{16}O_{27}P_6Ag_8 = 1569$
Calculated Ag 55.00; P 11.85 per ct.
Found Ag 55.98: P 11.94 per ct.

HEPTA-SILVER PHYTATE.

This salt is obtained when the octa-silver phytate, dissolved in dilute nitric acid, is precipitated with alcohol. The precipitate after filtering, washing and drying as before was analyzed. In appearance and properties it was identical with the octa salt.

For $C_6H_{17}O_{27}P_6Ag_7 = 1462$
Calculated Ag 51.64: P 12.72 per ct.
Found Ag 52.43: P 13.02 per ct.

DI-PYROPHOSPHORIC ACID ESTER OF INOSITE.

Dry pyrophosphoric acid 17.02 gm. (little over 9 mol.) was heated in a flask in an oil bath to about 200°C. and 5.4 gm. (3 mol.) dry inosite added. At this temperature the inosite dissolved at once forming a thick reddish-brown colored solution. After heating to 220° for a few minutes the flask was removed and allowed to cool. The reaction mixture was dissolved in 500 cc. of water, 20 cc. 5/N hydrochloric acid added and the whole boiled for about one hour. At the end of this time the excess of the pyrophosphoric acid has become changed to orthophosphoric acid and as such does not interfere with the precipitation of the barium salt of the ester with alcohol.

After cooling the above solution containing the new ester it was diluted to 1 liter with water, a solution of 40 gm. of barium chloride in water was added and the barium salt of the ester was then pre-

cipitated by adding 1 liter of alcohol. The resulting precipitate was filtered off at once and for the purpose of removing adhering inorganic phosphate was precipitated twice from 0.5 per ct. hydrochloric acid, in the presence of a small quantity of barium chloride, with alcohol and then twice from the same strength hydrochloric acid with alcohol. After finally filtering and thoroughly washing in 50 per ct alcohol, alcohol and ether it was dried in vacuum over sulphuric acid. The product so obtained was a white amorphous powder. In appearance it was very similar to the tribarium phytate and the barium salt of the tetra phosphoric acid ester of inosite except that when precipitated with alcohol the particles appeared coarser. On moist litmus paper it showed a strong acid reaction. It was readily soluble in dilute hydrochloric and nitric acids, less soluble in acetic acid, very slightly soluble in water and exceedingly soluble in 10 per ct. phytic acid. It was free from chlorine. Yield 11 8 gm.

After drying at 105° in vacuum over phosphorus pentoxide the substance was analyzed.

0.2617 gm. subst. gave 0.0421 gm. H_2O and 0.1016 gm. CO_2
0.2796 gm. subst. gave 0.0488 gm. H_2O and 0.1080 gm. CO_2
0.2566 gm. subst. gave 0.1495 gm. $BaSO_4$ and 0.1443 gm. $Mg_2P_2O_7$
Found: C 10.58: H 1.80: P 15.67: Ba 34.28 per ct.
C 10.53: H 1.95 per ct.

The substance was not yet pure being probably mixed with some monopyrophosphoric acid ester of inosite; at least the high carbon and low phosphorus points to such a conclusion.

It was hoped that the exceeding solubility of the substance in phytic acid might serve to separate these bodies. For this purpose the whole substance was dissolved in 20 cc. 10 per ct. phytic acid. On diluting with water a portion of the substance separated as a heavy granular powder. To complete the separation 100 cc. of water was added and then allowed to stand two days at room temperature. This precipitate was discarded; as analysis, after purifying by precipitating from 0.5 per ct. hydrochloric acid with alcohol, showed that it was still impure and only 0.9 gm. had been obtained. The great bulk of the substance was accordingly contained in the filtrate from the above. This filtrate was diluted to 300 cc. with water and then precipitated by adding 300 cc. alcohol. The voluminous white precipitate was filtered off, washed thoroughly in 50 per ct. alcohol and alcohol. For purification it was dissolved in 0.5 per ct. hydrochloric acid and precipitated by alcohol. After filtering and thoroughly washing in dilute alcohol until free from chlorine it was washed in alcohol and ether and dried in vacuum over sulphuric acid. The product so obtained was a pure white amorphous powder. On moist litmus paper it showed a strong

acid reaction. The solubility corresponded with that previously observed. Yield 7.7 gm.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0.2914 gm. subst. gave 0.0440 gm. H_2O and .1026 gm. CO_2.
0.1667 gm. subst. gave 0.0977 gm. $BaSo_4$ and 0.0960 gm. $Mg_2P_2O_7$.

For $C_6H_6(OH)_4\ O_2(P_2O_6HBa)_2 = 770.7$
Calculated C 9.34: H 1.55: P 16.08: Ba 35.64 per ct.
Found C 9.60: H 1.68: P 16.05: Ba 34.48 per ct.

The barium was found to be a little low but this is compensated for through a slightly high carbon content; moreover it is sometimes difficult to obtain amorphous salts of this kind which show closer agreement than the above. The analysis leaves no doubt that the substance was the barium salt of the ester in question. It will be noticed that the dipyrophosphoric acid ester is isomeric with the tetraphosphoric acid ester of inosite previously referred to. Lack of time has prevented the determination of the free alcoholic (OH) groups in the inosite ring in either of these compounds. Experiments along this line are contemplated, however.

That the reaction between the inosite and the pyrophosphoric acid actually occurred along the lines discussed above may be judged by the amount of water given off. To determine this point 0.36 gm. inosite (one mol.) and 1.06 gm. pyrophosphoric acid (3 mol.) both previously dried at 100° C. were heated in a small flask in oil bath at 200°–220° under the same conditions as in the above experiment. The water, which began to come over at a temperature of about 200°, was collected in a weighed calcium-chloride tube. The aqueous vapors were removed by means of the suction pump but no special effort was made to secure quantitative results. The water obtained weighed 0.0494 gm. whereas the quantity calculated for 2 mol. H_2O is 0.072 gm. The amount obtained is therefore only about 68 per ct. of the theory.

PREPARATION OF THE FREE DIPYROPHOSPHORIC ESTER.

The air-dried barium salt of the ester (4 gm.) was suspended in water and decomposed with a slight excess of sulphuric acid, the barium sulphate was removed and the solution precipitated with copper acetate. The copper salt was filtered off, thoroughly washed in water, suspended in water and decomposed with hydrogen sulphide. It was found impossible to remove the copper sulphide completely by filtration as it formed a colloidal solution, but by acidifying with a few drops of hydrochloric acid and heating to boiling the copper sulphide precipitated. After filtering, the filtrate was evaporated several times in vacuum for the removal of the hydrochloric

acid and finally dried in vacuum over sulphuric acid and potassium hydroxide until it was of a thick syrupy consistency. The product obtained was of the same appearance as phytic acid or the tetraphosphoric ester, viz.: a thick, light amber colored liquid. After drying at 105° the substance was analyzed.

0.1709 gm. subst. gave 0.0552 gm. H_2O and 0.0892 gm. CO_2.
0.1739 gm. subst. gave 0.1517 gm. $Mg_2P_2O_7$.

For $C_6H_6(OH)_4O_2$ $[P_2O_3(OH)_3]_2 = 500$
Calculated C 14.40: H 3.20: P 24.80 per ct.
Found C 14.23: H 3.61: P 24.31 per ct.

PROPERTIES OF THE FREE DIPYROPHOSPHORIC ESTER.

The concentrated aqueous solution is a thick, light amber colored syrup. On longer drying over sulphuric acid it becomes a hard and brittle hygroscopic mass.

The aqueous solution is of strong acid reaction and sharp acid taste.

With barium chloride no precipitate is produced either in the cold or on heating; alcohol or ammonia produces a white precipitate in this solution.

Calcium chloride gives no precipitate even on heating but alcohol causes in this solution a voluminous flocculent precipitate.

Calcium acetate produces at once a white precipitate sparingly soluble in acetic but readily soluble in mineral acids.

Magnesium acetate gives a white precipitate readily soluble in acids.

Ferric chloride gives a white or faintly yellowish precipitate very sparingly soluble in acids.

Barium acetate gives a white precipitate sparingly soluble in acetic acid but readily soluble in dilute hydrochloric or nitric acids.

Dilute silver nitrate does not cause a precipitate but concentrated silver nitrate gives a white precipitate.

With ordinary molybdate solution no precipitate is produced but neutral molybdate gives a white precipitate which slowly turns yellowish in color. On drying at 105° the substance turns very dark in color.

INOSITE FROM DI-PYROPHOSPHORIC ESTER.

The free ester, 0.65 gm., was heated with 20 cc. 5/N sulphuric acid in sealed tube to 150° for about three hours. The inosite was isolated by the usual method and crystallized from dilute alcohol after addition of ether. After recrystallizing from hot dilute alcohol, adding ether and allowing to stand several hours in the cold, the substance was obtained in small colorless crystals free from water of crystallization: Yield 0.18 gm. or 75 per ct. of the theory. The

air-dried, water-free substance melted at 221° C. (uncor.) and it gave the reaction of Scherer. Drying at 110° for one hour caused no loss of weight. On analysis the following results were obtained:
0.1634 gm. subst. gave 0.0981 H_2O and 0.2374 gm. CO_2.

For $C_6H_{12}O_6 = 180$
Calculated C 40.00: H 6.66 per ct.
Found C 39.62: H 6.71 per ct.

DI-INOSITE TRIPYROPHOSPHORIC ACID ESTER

This ester is formed when dry inosite is heated with excess of pyrophosphoric acid. The molecule of the new ester evidently consists of two molecules dipyrophosphoric acid esters of inosite joined by one molecule of pyrophosphoric acid.

Dry inosite 1.8 gm. (1 mol.) were heated with 10.7 gm. (6 mol.) pyrophosphoric acid under the same conditions as described for the dipyrophosphoric ester and it was isolated as the barium salt in exactly the same way. After precipitating five times from 0.5 per ct. hydrochloric acid with alcohol the product was finally obtained as a perfectly white amorphous powder. Its solubilities corresponded practically with those mentioned for the dipyrophosphoric acid ester and likewise it showed a strong acid reaction on moist litmus paper.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0 2086 gm. subst. gave 0.0296 gm. H_2O and 0.0614 gm. CO_2.
0.1412 gm. subst. gave 0.0879 gm. $BaSO_4$ and 0.0844 gm. $Mg_2P_2O_7$.
Found C 8.02: H 1.58: P 16.66: Ba 36.63 per ct.

This substance was then again precipitated twice from 0.5 per ct. hydrochloric acid with alcohol and after drying at 105° gave the following result on analysis:

0.2420 gm. subst. gave 0.0342 gm. H_2O and 0.0725 gm. CO_2.
0.2331 gm. subst. gave. 0.1422 gm. BaSO and 0.1384 gm. $Mg_2P_2O_7$.

For $C_{12}H_{22}O_{41}P_{10}Ba_5 = 1818$
Calculated C 7.92: H 1.21: P 17 05: Ba 37 73 per ct.
Found I C 8.02: H 1.58: P 16.66: Ba 36.66 per ct.
Found II C 8.17: H 1.58: P 16.55: Ba 35.90 per ct.

As repeated precipitations did not alter the composition it was undoubtedly a homogeneous compound. The barium was found to be too low, but as previously remarked, it is difficult to obtain these amorphous salts in absolutely pure form. The percentage of the base combined with the acid is apt to vary more or less, depending upon conditions. The analysis of the free ester leaves no doubt but that the substance was the compound in question.

PREPARATION OF THE FREE DI-INOSITE TRI-PYROPHOSPHORIC ACID ESTER.

The purified dry barium salt (1.5 gm.) was suspended in water, decomposed with slight excess of sulphuric acid, the barium sulphate removed and the solution precipitated with copper acetate. The copper salt was decomposed and the free ester obtained in exactly the same way as described for the di-pyrophosphoric ester.

In this case also the copper sulphide could be precipitated only after the solution had been acidified with hydrochloric acid. For the removal of the hydrochloric acid the filtrate was evaporated several times in vacuum with water and finally dried in vacuum over sulphuric acid and potassium hydroxide. The product, like the previous compound, was a thick, light amber colored syrup. For analysis it was dried at 105° C.

0.1607 gm. subst. gave 0.0466 gm. H_2O and 0.0744 gm. CO_2.
0.1083 gm. subst. gave 0.1030 gm. $Mg_2P_2O_7$.

For $C_{12}H_{32}O_{41}P_{10} = 1142$
Calculated C 12.60: H 2.80: P 27.14 per ct.
Found C 12 62: H 3.24: P 26.51 per ct.

PROPERTIES OF THE DI-INOSITE TRI-PYROPHOSPHORIC ESTER

The properties of this ester agree in the main with those mentioned of the di-pyrophosphoric ester. The concentrated solution of the ester forms a thick, light amber colored syrup which on longer drying in desiccator becomes brittle and hygroscopic. The aqueous solution is of strong acid reaction and pleasant acid taste. The precipitates produced with calcium, magnesium, silver and iron salts are identical with those given by the di-pyrophosphoric ester.

Barium chloride produces at once a white precipitate sparingly soluble in acetic but readily soluble in dilute hydrochloric and nitric acids.

Ordinary molybdate solution produces a white precipitate which does not turn yellowish in color, being in this respect identical with phytic acid.

Neutral molybdate solution causes at first a voluminous white precipitate which redissolves almost immediately. The addition of a few drops of the ordinary acid molybdate to this solution and scratching with a glass rod causes the separation of long white needle-shaped crystals. The crystals and the precipitate caused by the ordinary molybdate solution are readily soluble in ammonia.

On drying at 105° the substance turns very dark in color.

Lack of material prevented the hydrolysis of this ester and the isolation of inosite as one of the products of decomposition had therefore to be omitted.

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GENEVA, N. Y.

THE ORGANIC-PHOSPHORIC ACID COMPOUND OF WHEAT BRAN

R. J. ANDERSON

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TECHNICAL BULLETIN No. 22.

THE ORGANIC-PHOSPHORIC ACID COMPOUND OF WHEAT BRAN: PRELIMINARY REPORT.*

(THIRD PAPER ON PHYTIN.)

R. J. ANDERSON.

SUMMARY.

In the examination of the organic-phosphoric acid compound of wheat bran none of the characteristic salts of phytic acid could be isolated.

The purified barium salts of the compound corresponded to the following formulas: $C_{25}H_{55}O_{54}P_9Ba_5$ and $C_{20}H_{45}O_{49}P_9Ba_5$.

Attempts to isolate the free acid corresponding to the first salt did not succeed. From both salts the same acid, corresponding to that of the second salt, $C_{20}H_{55}O_{49}P_9$ was obtained. This acid is apparently formed from the first by the splitting off of the elements of one pentose.

Crystalline salts of the acid $C_{20}H_{55}O_{49}P_9$ with inorganic bases could not be obtained. A crystalline brucine salt $C_{20}H_{55}O_{49}P_9(C_{23}H_{26}O_4N_4)_{10}+30H_2O$ was easily formed.

Since all the purified barium salts prepared under different conditions, either from the previously isolated crude substance or from the bran extract itself, could all finally be changed into salts of the acid $C_{20}H_{55}O_{49}P_9$ under liberation of reducing substances, the conclusion seems justified that this acid is the only organic-phosphoric-acid present and that wheat bran does not contain phytin.

INTRODUCTION.

In the last two papers dealing with the chemistry of phytin[1] various salts of phytic acid with inorganic bases have been described as well as various phosphoric and pyrophosphoric acid esters of inosite. In connection with the above work the subject of the organic phosphorus compound of wheat bran was taken up.

Patten and Hart[2] had shown that wheat bran contains an organic-phosphorus body which on cleavage with 30 per ct. sulphuric acid in a sealed tube gave inosite as one of the products of decomposition. They also obtained an acid from a 0.2 per ct. hydrochloric acid extract of bran which on analysis gave results corresponding very closely with the theoretical composition of phytic acid or, as the substance was then called, " anhydro-oxymethylene di-phosphoric acid ".

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[1] *Jour. Biol. Chem.* 11: 471, and 12: 97; and Technical Bulls. 19 and 21 of this Station.

[2] *Amer Chem. Jour.* 31: 566. 1904.

These results led the above authors to believe that the substance which they had isolated was identical with the organic-phosphorus compound described by Palladin,[1] Schulze and Winterstein,[2] and later by Winterstein,[3] and which was finally obtained in pure form by Posternak,[4] who gave the substance the name " phytin ".

The above substance, isolated by Patten and Hart and which they assumed to be phytin, has since then been regarded as such by other investigators, among whom may be mentioned Suzuki and Yoshimura,[5] Neuberg,[6] and Forbes.[7] These authors do not, however, report any complete analyses of the substance.

Since the investigation of the organic-phosphorus compound of wheat bran by Patten and Hart, several feeding experiments, some of which have not yet been published, to determine the physiological effect of phytin have been carried out at this institution by Dr. Jordan. In these experiments it has been found that the effect of pure phytin salts has been decidedly different from that obtained by feeding varying quantities of washed and unwashed wheat bran.[8] These anomalous results could not be explained on the assumption that only " phytin " was removed from the bran by washing. What still more complicated the problem was the fact that previous work had shown that very little besides phosphorus compounds and inorganic bases had been removed from the wheat bran in the process of washing or leaching.[9]

In the hope of throwing some light on this subject, the chemical investigation of the products removed from wheat bran by washing it in dilute acid was again taken up. The chief object was to isolate and identify the organic-phosphorus body and to determine what bases were associated with it.

If ordinary wheat bran be extracted with 0.2 per ct. hydrochloric acid and the resulting filtered extract precipitated with alcohol, a body is obtained which, after repeated precipitations from 0.2 per ct. hydrochloric acid with alcohol, shows a relatively uniform composition. The composition varies somewhat, depending upon the conditions under which the substance is prepared, but on an average it has been found to be about as follows: C 21.0, H 3.5, P 14.0 per ct. The substance also contains calcium, magnesium, potassium and sodium in varying amounts, together with traces of iron, and it always contains nitrogen varying from 2.1 to 0.4 per ct. The nitrogen, however, is not present as ammonia.

[1] *Ztschr. Biol.* **31**: 199. 1894.
[2] *Ztschr. physiol. Chem.* **22**: 90.
[3] *Ber. deut. Chem. Ges.* **30**: 2299.
[4] *Rev. Gen. Bot.* **12**: 5 and 65 (1900) and *Compt. Rend.* **137**: 202, 337 and 439 (1903).
[5] *Bull. Coll. Agric.* (Tokyo) **7**: 498.
[6] *Biochem. Ztschr.* **16**: 405.
[7] Ohio Agr. Exp. Sta. Bull. 215.
[8] *Amer. Jour. Physiol.* **16**: 268. 1906.
[9] *Ibid.* **16**: 274 and 304. 1906.

This compound reduces Fehling's solution on boiling. It gives reactions with orcine and phloroglucine and yields furfurol when distilled with 12 per ct. hydrochloric acid It was first described by Patten and Hart,[1] although they do not mention the above reactions. As previously stated, they considered it identical with phytin, although according to their analysis it had the following composition: C 18.52, H 3.83, P 16.38, Ca 1.13, Mg 5.80, K 2.60, N 0.37 per ct.

It will be noticed at once that for a salt of phytic acid the above compound has about 10 per ct. *too much carbon* and about 6 per ct. *too little phosphorus.*

After isolating and purifying some of the substance, as will be described in the experimental part, a product was finally obtained which had the composition mentioned above, viz: C 21.0 per ct., etc. It was believed at first that it was an impure phytin compound, probably associated with some carbohydrate group and some complex nitrogen-containing body. All attempts to prepare any of the characteristic salts of phytic acid from the substance have failed. We have found, however, that it is possible by the action of barium hydroxide to separate the substance into two constituents: one an organic-phosphoric acid free from nitrogen, and a second body containing nitrogen.

The nitrogen-containing compound also contains phosphorus in organic combination. It has, however, not been obtained in pure form. It has not been analyzed and its nature is at present entirely unknown.

The barium salt of the nitrogen-free body corresponds to the formula $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$. The isolation of the free acid, $C_{25}\ H_{65}\ O_{54}\ P_9$, corresponding to the above barium salt, has not succeeded. Attempts to isolate it led to an organic-phosphoric acid, lower in carbon and higher in phosphorus, of the composition $C_{20}\ H_{55}\ O_{49}\ P_9$. In the process of isolation apparently the elements of one pentose are split off:

$$C_{25}\ H_{65}\ O_{54}\ P_9 - C_5\ H_{10}\ O_5 = C_{20}\ H_{55}\ O_{49}\ P_9.$$

If, in the preparation of the above barium salt from the crude substance, the solution is allowed to stand in contact with dilute hydrochloric acid for any length of time a barium salt is obtained corresponding to the second acid, $C_{20}\ H_{45}\ O_{49}\ P_9\ Ba_5$ from which salt the free acid may be generated.

The barium salt, $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$, yields furfurol on distillation with 12 per ct. hydrochloric acid but the salt $C_{20}\ H_{45}\ O_{49}\ P_9\ Ba_5$ does not do so.

The acid, $C_{20}\ H_{55}\ O_{49}\ P_9$, apparently represents the nucleus of the molecule of the organic-phosphorus compound, as it has been found impossible to obtain any simpler organic-phosphoric acid from it by treatment with acids.

[1] *Loc. cit.*

On boiling with normal sulphuric acid at ordinary pressure it is slowly decomposed with formation of phosphoric acid and *reducing bodies*, apparently carbohydrates, as the solution reduces Fehling's solution and gives reactions with orcine and phloroglucine; but no trace of inosite could be isolated. The unchanged portion isolated from the reaction mixture has exactly the same composition as it had before boiling, which indicates that the molecule is gradually broken up into reducing bodies and phosphoric acid without suffering any intermediate or partial decomposition.

On heating the substance in a sealed tube with 5/N sulphuric acid the cleavage appears to go in another direction; for in this case 90 per ct. of the total carbon was recovered in the form of inosite and absolutely no reducing bodies were present in the reaction mixture. No explanation can be offered at this time concerning this peculiar behavior towards sulphuric acid under ordinary pressure and in a sealed tube.

It is evident that this compound is *not phytin*. The only similarity between these substances is found in that they are both organic-phosphoric acids and that when heated in a sealed tube with acids they yield inosite as one of the products of decomposition. Whether this new compound contains the inosite as such or whether it is only formed in the process of decomposition cannot be definitely determined at this time. However, if it were a complex compound of inosite and phosphoric or pyrophosphoric acid, the isolation of inosite should be possible after cleavage with dilute acid at ordinary pressure. As has been stated, this cannot be done and, moreover, the empirical formula of the substance can hardly be brought into accord with any inosite compound.

The substance is probably similar to, if not identical with, the glucophosphoric acid described by Levene.[1] The same author[2] also described an organic-phosphoric acid compound isolated from hemp seed which gave reactions for pentose or glucoronic acid. Since phytin does not give these reactions, as stated by Neuberg,[3] it is very probable that the products examined by Levene were of the same nature as that described in this paper.

The chief support of the assumption of Patten and Hart[4] that the organic-phosphorus compound of wheat bran was phytin was no doubt based upon the fact that they had obtained a substance from dilute hydrochloric-acid extract of bran which corresponded closely in composition with that required for the " anhydro-oxymethylene di-phosphoric acid " of Posternak.

Serious objection, however, must be raised against their method of isolating this substance in so far as they made absolutely no

[1] *Jour. Amer. Chem. Soc* 24: 190 (1902), and *Amer. Jour. Physiol.* 8: XI (1903).
[2] *Biochem. Ztschr.* 16: 399.
[3] *Ibid* 16: p. 405.
[4] *Loc. cit.*

effort to remove *inorganic phosphates*. From the work of Hart and Andrews[1] they believed themselves justified in considering the inorganic soluble phosphates present in wheat bran as a negligible quantity. While we cannot enter into any discussion of the above work here, it is to be noted that in the determination of the soluble inorganic phosphates in plant constituents Hart and Andrews extracted the material with 0.2 per ct. hydrochloric acid for 15 minutes and determined the inorganic phosphorus in the filtered extract by precipitating with nearly neutral ammonium molybdate. By this method they found 0.036 per ct. inorganic phosphorus in wheat bran. The total amount of phosphorus compounds soluble in 0.2 per ct. hydrochloric acid was found to be equivalent to 0.951 per ct. phosphorus.

The inorganic phosphorus found by the above authors is therefore equal to 3.78 per ct. of the total phosphorus soluble in 0.2 per ct. hydrochloric acid.

Suzuki and Yoshimura[2] report phosphorus determinations in wheat bran. They found 0.638 per ct. phosphorus soluble in 0.2 per ct. hydrochloric acid; of this, 0.050 per ct. was found to be inorganic, and 0.579 per ct. organic. The inorganic phosphorus found in this case is therefore equal to 8.63 per ct. of the total phosphorus soluble in 0.2 per ct. hydrochloric acid.

The work of Forbes and associates[3] seems to show that the time allowed by Hart and Andrews, 15 minutes, is not sufficient for complete extraction and that neutral molybdate solution is not suitable for precipitation in all cases; that when three hours is allowed for extraction a considerably larger amount of inorganic phosphorus is obtained.

In the preparation of the " phytin " products from wheat bran Patten and Hart[4] do not mention any definite time allowed for extraction but only state that " the bran was extracted for several hours with 0.2 per ct. hydrochloric acid," apparently therefore a longer time than allowed by Hart and Andrews in their determinations of inorganic-phosphorus.

When wheat bran is digested for several hours in 0.2 per ct. hydrochloric acid we have found that the resulting extract always contains a considerable quantity of inorganic phosphates. Quantitative determinations have, however, not been carried out and we are at present unable to state whether the inorganic phosphates were present in the bran originally or if they have been formed by hydrolysis of the organic-phosphorus compounds, but we purpose to take up this phase of the subject later.

[1] N. Y. Agr. Exp. Station Bull. 238. 1903.
[2] *Bull. Coll. Agr.* (Tokyo) 7: 498.
[3] *Loc. cit.*
[4] *Loc. cit.*

On precipitating a bran extract, prepared as indicated above, with any of the usual reagents for the isolation of the organic-phosphorus compound the inorganic phosphates are more or less completely precipitated at the same time. In order to remove these inorganic phosphates we have found it necessary to repeatedly precipitate the substance from 0.2 or 0.5 per ct. hydrochloric acid with alcohol. In other words the substance has been re-precipitated until the dilute nitric acid solution of the resulting product does not give any immediate reaction with ammonium molybdate. The slight amount of phospho-molybdate precipitated from the solution on longer standing is no doubt due to cleavage rather than to admixed inorganic phosphates.

In order to determine if any barium salt of different composition from those discussed above could be prepared directly from wheat bran extract, the following experiment was carried out: The 0.2 per ct. hydrochloric acid extract of bran prepared as before was precipitated with barium chloride and alcohol. The substance was purified by precipitating from 0.5 per ct. hydrochloric acid solution with alcohol until it gave no immediate reaction with ammonium molybdate (compare experimental part).

Analysis showed that this compound contained a higher percentage of carbon and lower of phosphorus than the barium salt prepared from the previously isolated crude substance and it gave a larger amount of furfurol on distillation with 12 per ct. hydrochloric acid. By treating this compound with dilute sulphuric acid for a short time some reducing body was split off and the organic-phosporus substance finally isolated from the reaction mixture corresponded in composition with the barium salt first prepared, viz., $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$. This compound is, however, easily transformed into $C_{20}\ H_{55}\ O_{49}\ P_9$ as has already been shown.

Since we have been unable to isolate any compound from wheat bran corresponding in composition to a salt of phytic acid we have come to the conclusion that wheat bran *does not contain phytin* and that the above compound $C_{20}\ H_{55}\ O_{49}\ P_9$ is the only organic-phosphoric acid existing in bran. It appears, however, that in its natural condition in the bran one or more as yet unidentified reducing bodies, yielding furfurol on distillation with hydrochloric acid, and which are easily split off by the action of dilute acids, are loosely bound to this nucleus.

The so-called "anhydro-oxymethylene di-phosphoric acid" analyzed by Patten and Hart was undoubtedly a mixture of the above compound and free phosphoric acid. This seems the more probable as they had not made any effort to remove inorganic phosphates in the preparation of their acid.

The empirical formulas suggested in this paper are of course purely tentative. We are now preparing larger quantities of the

substance from wheat bran and hope shortly to be able to report further concerning this compound. Various other cereals and feeding stuffs are also being examined to determine if they contain phytin or if this other organic-phosphoric acid compound is present.

EXPERIMENTAL.

PREPARATION OF THE ORGANIC-PHOSPHORUS COMPOUND FROM WHEAT BRAN.

The bran was digested with 0.2 per ct. hydrochloric acid over night and the extract after straining and filtering was precipitated with alcohol according to the method of Patten & Hart (loc. cit.). The resulting precipitate was purified by precipitating five times from 0.2 per ct. hydrochloric acid with alcohol. From 500 grams bran 2.5 grams substance was obtained as a white amorphous non-hygroscopic powder. It is readily soluble in its own weight of water, forming a thick light-amber-colored solution of pleasant and characteristic but faint acid odor. The substance reduces Fehling's solution on boiling and it gives the orcine and phloroglucine reactions. The aqueous solution is of acid reaction on litmus paper. It is precipitated by alkalies and solutions of salts of other metals. Warmed with dilute nitric acid and ammonium molybdate it does not give any immediate precipitate but on standing for several hours a trace of yellow phosphomolybdate is precipitated.

After drying at 120° the substance was analyzed.

0.1642 gram substance gave 0.0522 gm. H_2O and 0.1285 gm. CO_2.

0.0860 gram substance gave 0.0428 gm. $Mg_2P_2O_7$ for P.

0.1720 gram substance gave 0.0264 gm. $Mg_2P_2O_7$ for Mg.

0.1720 gram substance gave 0.0054 gm. CaO.

0.3897 gram substance gave 0.0085 gm. N (Kjeldahl).

The substance contained only a very small quantity of K.

Found C 21.34, H 3.55, P 13.87, Ca 2.24, Mg 3.35, N 2.18 per ct.

A larger quantity of the product was then prepared by extracting 3 kg. of bran. After isolating and purifying in the same way as before 47 grams substance was obtained or about 1.5 per ct. of the weight of the bran used. In appearance and properties it was identical with the foregoing.

After drying at 105° in vacuum over phosphorus pentoxide the substance was analyzed.

0.2444 gram substance gave 0.0710 gm. H_2O and 0.1940 gm. CO_2.

0.3936 gram substance gave 0.0149 gm. CaO and 0.0861 $Mg_2P_2O_7$.

0.3936 gram substance gave 0.0623 gm. K_2PtCl_6.

Phosphorus and nitrogen determinations were not made.

Found C 21.64, H 3.25, Ca 2.70, Mg 4.77, K 2.54 per ct.

The substance was again precipitated from 0.2 per ct. hydrochloric acid, washed and dried as before when the following results were obtained on analysis:

0.1934 gram substance gave 0.0639 gm. H_2O and 0.1523 gm. CO_2.

0.3313 gram substance gave 0.0131 gm. CaO and 0.0717 gm. $Mg_2P_2O_7$ for Mg.

0.3313 gram substance gave 0.0444 gm. $K_2\ PtCl_6$.

0.1656 gram substance gave 0.0857 gm. $Mg_2P_2O_7$ for P.

0.4639 gram substance gave 0.0058 gm. N (Kjeldahl).

Found C 21.47, H 3.69, P 14.42, Ca 2.82, Mg 4.72, K 2.15, N 1.25 per ct.

Sodium was not determined but qualitative tests showed that it was present.

The reprecipitated substance, 0.3887 gm., distilled with 12 per ct. HCl gave 0.0367 gm. phloroglucid.

As the composition did not change by reprecipitation it was deemed sufficiently pure to use in the subsequent experiments.

It was thought at first that this substance might be phytin mixed with some carbohydrate and some basic nitrogen body. In the hope of separating these and to obtain pure compounds the substance was treated with barium hydroxide and the resulting barium salt purified as follows:

PREPARATION OF THE BARIUM SALT.

Five grams of the substance were dissolved in 10 cc. of water and the solution diluted to 200 cc. with water. Barium hydroxide was then added until distinctly alkaline and the mixture heated nearly to boiling. It was then filtered hot and washed with hot water, the filtrate being reserved for examination.

The washed barium precipitate was dissolved in just sufficient 0.5 per ct. hydrochloric acid, filtered, again precipitated with barium hydroxide, the resulting precipitate dissolved by the careful addition of dilute hydrochloric acid and then precipitated by the addition of a like volume of alcohol. The substance was filtered, washed in dilute alcohol, again dissolved in 0.5 per ct. hydrochloric acid and precipitated in the same manner as before. These operations were repeated four times. It was then dissolved in the same strength hydrochloric acid, precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a perfectly white amorphous powder. Yield 3.9 grams.

The substance was slightly soluble in boiling water. On cooling, however, it does not crystallize out and on concentrating in vacuum it separates in an amorphous form. Alcohol also produces a white amorphous precipitate. Various other methods were tried to obtain the substance in crystalline form but without success. On moist

litmus paper it shows a strong acid reaction. It was free from nitrogen.

As it was found impossible to crystallize the substance it was analyzed directly after drying at 130°.

0.2564 grams substance gave 0.0604 gm. H_2O and 0.1314 gm. CO_2.

0.2903 grams substance gave 0.1544 gm. $BaSO_4$ and 0.1324 gm. $Mg_2P_2O_7$.

Found C 13.97, H 2.63, P 12.71, Ba 31.29 per ct.

Of this substance 1.2124 gm. was distilled with 12 per ct. HCl when 0.0053 gm. phloroglucid was obtained.

The composition of the above salt is entirely different from that of a barium phytate. The relation of the numbers found lead to the empirical formula $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$=2185.

Calculated for this C 13.73, H 2.51, P 12.76, Ba 31.44 per ct.

EXAMINATION OF THE FILTRATE FROM THE ABOVE COMPOUND AFTER PRECIPITATING WITH BARIUM HYDROXIDE.

The filtrate was of light amber color. The excess of barium hydroxide was removed with carbon dioxide, filtered and the filtrate concentrated on the waterbath; again filtered from traces of barium carbonate and then dried in vacuum over sulphuric acid. There remained a small quantity of a yellowish amber-colored, somewhat gummy mass. It contained a large quantity of nitrogen. It did not reduce Fehling's solution and gave only a faint biuret reaction.

The substance is readily soluble in water and is again precipitated by alcohol but it is not precipitated by tannic acid. The aqueous solution acidified with nitric acid gives no reaction with ammonium molybdate.

After combustion the ash was found to contain potassium, sodium and phosphorus. When the crude substance is treated by the Van Slyke method for amino nitrogen a small quantity of nitrogen is liberated.

Lack of time has prevented the further examination of this body and it has not been isolated in pure form.

ISOLATION OF THE FREE ACID FROM THE FOREGOING BARIUM SALT.

The barium salt (3.2 grams dry substance) was suspended in 100 cc. of water and decomposed with a slight excess of dilute sulphuric acid; the barium sulphate was removed by filtration and the filtrate precipitated with excess of copper acetate. The copper salt was filtered, thoroughly washed in water, suspended in water and decomposed with hydrogen sulphide. The copper sulphide was filtered off and the filtrate concentrated in vacuum to small bulk and finally dried in vacuum over sulphuric acid until it was of a thick syrupy consistency. After drying at 100° to constant weight the substance was analyzed.

0.2907 gram substance gave 0.1052 gm. H_2O and 0.1855 gm. CO_2.
0.1787 gram substance gave 0.0642 gm. H_2O and 0.1144 gm. CO_2.
0.1816 gram substance gave 0.1331 gm. $Mg_2P_2O_7$.
Found I C 17.40, H 4.04, P 20.43 per ct.
II C 17.46, H 4.02 per ct.
These results lead to the empirical formula C_{20} H_{55} O_{49} P_9.
Calculated for C_{20} H_{55} O_{49} P_9=1358.
C 17.67, H 4.05, P 20.54 per ct.

This compound differs in composition from the barium salt from which it was prepared by C_5 H_{10} O_5; in other words by the elements of one pentose. This had probably been split off in the decomposition of the barium salt with the dilute sulphuric acid or else by the copper acetate, and if so should be found in the filtrate after the copper salt of the acid had been removed. The filtrate was therefore examined as follows: The copper was removed by hydrogen sulphide and the filtrate, after boiling off excess of H_2S, was precipitated with excess of barium hydroxide; filtered, and the barium precipitated quantitatively with sulphuric acid and the resulting filtrate evaporated to small bulk in vacuum. The solution was then found to reduce Fehling's solution on boiling and ammoniacal silver nitrate was also reduced. Unfortunately the substance obtained was too small for any further examination. There is, however, absolutely no doubt that a reducing body was present and this was most likely the above mentioned pentose.

PROPERTIES OF THE FREE ACID,

C_{20} H_{55} O_{49} P_9.

Dried in the desiccator it forms first a light-amber-colored thick syrup which on continued drying forms a thick sticky mass. It is very soluble in water and also readily soluble in alcohol from which solution it is thrown out by the addition of ether as a white precipitate which collects on the sides of the test tube in small oily drops.

The aqueous solution has a strong acid reaction and a pleasant sharp acid taste and it gives the following reactions:

Magnesium acetate does not give a precipitate but the addition of calcium acetate, barium chloride or alcohol causes in this solution a white precipitate.

Silver nitrate does not produce any precipitate but the addition of alcohol gives a white amorphous precipitate of the silver salt.

It is not precipitated by barium or calcium chlorides but the acetates of these metals and their hydroxides give white amorphous precipitates which are soluble in acetic and mineral acids.

Ferric chloride causes a white precipitate which is readily soluble in dilute hydrochloric or nitric acids.

The alkali salts are very soluble in water but in these solutions salts of the alkaline earths or the heavy metals produce white

precipitates. The addition of alcohol also produces white precipitates.

The ordinary molybdate solution does not give any precipitate in dilute solutions of the acid; in concentrated solutions a yellowish white precipitate is obtained. On acidifying with nitric acid and heating, the yellow phospho-molybdate is slowly precipitated.

The aqueous solution of the acid is only incompletely precipitated by magnesia mixture. A slight white-colored amorphous precipitate is obtained but the addition of alcohol produces a voluminous white precipitate. This product is, however, not a pure salt as shown by the following results which were obtained on analysis of the dried precipitate:

Found Mg 11.29, N 2.40, P 16.45 per ct., which numbers do not agree with any formula for a pure magnesium ammonium salt of the above acid.

A larger quantity of the barium salt was prepared by treating 25 grams of the substance with barium hydroxide and purifying the barium salt in the same way as before, except that after precipitating the dilute hydrochloric-acid solution with alcohol the mixture was allowed to stand for several days.

After drying at 125° the following results were obtained on analysis:

C 12.05, H 2.46, P 13.83, Ba 32.19 per ct.

C 11.85, H 2.32, P 13.82, Ba 32.08 per ct.

Although the barium is found somewhat low this salt corresponds to the penta-barium salt of the acid $C_{20}\ H_{55}\ O_{49}\ P_9$.

For $C_{20}\ H_{45}\ O_{49}\ P_9\ Ba_5 = 2035$.

Calculated C 11.79, H 2.21, P 13.71, Ba 33.76 per ct.

The free acid prepared from this salt by the same method as before gave the following results on analysis after previously drying at 130°.

C 16.91, H 3.96, P 20.88 per ct.

C 16.91, H 3.84.

It appears then that the substance $C_{25}\ H_{65}\ O_{45}\ P_9$ is very sensitive to acids and that when it is kept in contact with even dilute acids for any length of time the elements of one pentose, $C_5\ H_{10}\ O_5$, are split off.

BRUCINE SALT OF THE ACID,

$$C_{20}\ H_{55}\ O_{49}\ P_9.$$

While it was impossible to obtain any crystalline salts of the above acid with inorganic bases it gave a crystalline brucine salt of the formula $C_{20}\ H_{55}\ O_{49}\ P_9\ (C_{23}\ H_{26}\ O_4\ N_2)_{10} + 30\ H_2O$.

About one gram of the acid was dissolved in a small quantity of water and brucine was then added until the solution showed a slight alkaline reaction. After diluting the solution with 150 cc. alcohol and 30 cc. chloroform, ether was added until a slight permanent turbidity remained. On standing for several days at room tempera-

ture in a well-closed Erlenmeyer flask the substance separated slowly in long white silky needle-shaped crystals.

In the absence of chloroform or in more concentrated solutions only amorphous white precipitates are obtained.

The crystals were filtered off and washed in a mixture containing equal parts of alcohol and ether and finally in ether and dried in the air. Yield about 0.5 grams.

The substance is very soluble in water, readily soluble in alcohol, but insoluble in ether or chloroform.

Heated in a capillary tube the substance melts at 196°–198°, but the melting point is not sharp. On moist litmus paper it shows a strong acid reaction. It loses weight on drying corresponding to 30 H_2O. The dried substance was analyzed.

0.1364 gm. substance lost 0.0124 gm. H_2O and 0.1308 gm. substance lost 0.0118 gm. H_2O.

0.1240 gm. substance gave 0.0694 gm. H_2O and 0.2557 gm. CO_2.

0.1383 gm. substance gave 0.0233 gm. $Mg_2P_2O_7$.

0.1190 gm. substance gave 6.1 cc. nitrogen by 16° and 746 mm.

For $C_{20}\ H_{55}\ O_{49}\ P_9\ (C_{23}\ H_{26}\ O_4\ N_2)_{10} = 5298$.

Calculated C 56.62, H 5.94, P 5.26, N 5.28 per ct.

Found C 56.24, H 6.26, P 4.69, N 5.86 per ct.

Calculated for 30 H_2O 9.24 per ct.; found 9.09 and 9.02 per ct.

ACTION OF DILUTE SULPHURIC ACID ON THE BARIUM SALT.

Five grams of the air-dried salt, $C_{20}\ H_{45}\ O_{49}\ P_9\ Ba_5$, were boiled for one hour under a reflux condenser with 100 cc. N/1 $H_2\ SO_4$. The reaction mixture was precipitated with slight excess of barium hydroxide, filterd and washed with water. The filtrate was examined as mentioned below.

The barium precipitate was shaken up with 300 cc. 0.5 per ct. hydrochloric acid and the insoluble portion filtered off. To the filtrate was added an equal volume of alcohol and the white flocculent precipitate filtered off and washed in dilute alcohol. It was again dissolved in 0.5 per ct. hydrochloric acid, precipitated with alcohol, filtered, washed free of hydrochloric acid with dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. Yield 2 grams. The product was a white amorphous powder. After drying at 120° the following results were obtained on analysis:

Found C 11.64, H 2.25, P 13.95, Ba 33.26 per ct.

This corresponds exactly with the composition of the substance before treatment with N/1 $H_2\ SO_4$. It is apparent therefore that no partial decomposition takes place.

EXAMINATION OF THE FILTRATE FROM ABOVE.

The excess of barium hydroxide was removed with carbon dioxide and, after filtering, the filtrate was concentrated in vacuum at a

temperature of 35°–40° to small bulk, again filtered and finally dried in vacuum over sulphuric acid. There remained 0.08 gram of a slightly amber-colored amorphous mass of weak acid reaction on litmus paper and a slight acid taste. The aqueous solution reduced Fehling's solution strongly on boiling, and it also gave the orcine and phloroglucine reactions. The small quantity of the substance prevented any further examination.

In another case $2\frac{1}{2}$ grams of the same barium salt were boiled with 100 cc. N/1 H_2SO_4 under reflux condenser for ten hours. After treating in the same way as above 0.3 gm. unchanged substance was obtained and the filtrate showed exactly the same properties as mentioned above. That is, it reduced Fehling's solution strongly on boiling, gave the reactions with orcine and phloroglucine, but attempts to isolate inosite failed, as no trace of this substance could be found.

PREPARATION OF INOSITE FROM THE BARIUM SALT.

Of the same barium salt (C_{20} H_{45} O_{49} P_9 Ba_5) 2.73 grams and 20 cc. 5/N H_2SO_4 were heated in a sealed tube for three hours to 160°. There was no pressure noticeable on opening the tube. Some free carbon had separated and the solution was of light-brown color. The neutralized solution did not reduce Fehling's solution. The inosite was isolated in the usual way, and after re-crystallizing from dilute alcohol and ether was obtained in needle-shaped crystals free from water of crystallization. It gave the reaction of Scherer and melted at 220.5° (uncorrected), which leaves no doubt but that the substance was pure inosite. Yield 0.73 gm., which is equal to 90 per ct. of the total carbon present in the barium salt used. The air-dried substance was analyzed.

0.1649 gm. substance gave 0.1038 gm. H_2O and 0.2406 gm. CO_2.
0.1323 gm. substance gave 0.0815 gm. H_2O and 0.1931 gm. CO_2.
For C_6 H_6 $(OH)_6 = 180$.
Calculated C 40.00 H 6.66 per ct.
Found C 39.80 H 7.04 per ct.
C 39.80 H 6.89 per ct.

The 0.2 per ct. hydrochloric acid extract of bran contains some dissolved proteins. On precipitating with alcohol these are thrown down together with the phosphorus compounds. Their presence makes the subsequent purification difficult, especially the filtrations, because the proteins have been rendered more or less insoluble and form a fine slimy mass which clogs the filter paper to such an extent as to make filtration even by suction extremely tedious.

In order to obviate this the suggestion was made by Dr. Jordan to first precipitate the bran extract with tannic acid.

The addition of tannic acid was found to cause a voluminous and very fine precipitate which after standing a short time becomes

coarser and may then be easily removed by simple filtration. The resulting filtrate is nearly colorless or of light amber color. Alcohol produces in this solution a nearly colorless precipitate which is much more easily purified than the product obtained without first precipitating with tannic acid.

With only this modification some of the substance was prepared from wheat bran. It was found, however, to differ slightly in composition from that obtained by the first method. On analysis the following results were obtained: C 19.51, H 3.09, P 15.23, Ca 0.38, Mg 7.35, K 2.75, N 0.57 per ct.

On treating this substance with barium hydroxide and purifying the resulting precipitate in the same way as before the same barium salt was obtained:

For C_{25} H_{55} O_{54} P_9 Ba_5 = 2184.

Calculated C 13.73, H 2.51, P 12.76, Ba 31.44 per ct.

Found C 13.00, H 2.46, P 12.47, Ba 33.00 per ct.

The difference in composition of the crude substance must therefore be due to the smaller amount of the nitrogen-containing body which this preparation was found to hold. In the analysis of the crude substance only 0.57 per ct. nitrogen was found, whereas the first preparation had four times, and the second preparation two times as much.

ISOLATION OF THE SUBSTANCE AS A BARIUM SALT DIRECTLY FROM THE BRAN EXTRACT.

The bran was digested with 0.2 per ct. hydrochloric acid over night The strained extract was precipitated with tannic acid, filtered, and a solution of barium chloride added, which caused a small precipitate to separate. An equal volume of alcohol was then added. After settling, the precipitate was filtered and purified as follows: The substance was dissolved in 0.5 per ct. hydrochloric acid, precipitated with barium hydroxide in excess, filtered, again dissolved in the same strength hydrochloric acid and then precipitated with alcohol. It was then precipitated a second time with barium hydroxide, and after that precipitated from 0.5 per ct. hydrochloric acid with alcohol until the product did not give any reaction with ammonium molybdate.

A white amorphous powder was finally obtained. On moist litmus paper it showed a strong acid reaction. After drying at 105° in vacuum over phosphorus pentoxide it was analyzed:

0.2870 gm. substance gave 0.0630 gm. H_2O and 0.1584 gm. CO_2.

0.3066 gm. substance gave 0.0653 gm. H_2O and 0.1700 gm. CO_2.

0.2632 gm. substance gave 0.1437 gm. $BaSO_4$ and 0.1020 gm. $Mg_2P_2O_7$.

Found I C 15.05, H 2.45, P 10.80, Ba 32.12 per ct.

II C 15.12, H 2.38.

Distilled with 12 per ct. HCl 0.4736 gm. substance gave 0.071 gm. phloroglucid.

As will be noticed, this compound contains a considerably larger percentage of carbon than any of the previous preparations and a correspondingly low percentage of phosphorus. Calculated on the same basis as before, it would correspond to a molecule with C 30 or C 32. By acting upon this compound with dilute sulphuric acid some reducing body is split off and the salt $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$ results, identical with that obtained in the first case from the crude substance.

One gram of the above barium salt was digested for about ten minutes with 20 cc. normal sulphuric acid and heated nearly to boiling. It was then precipitated with excess of barium hydroxide and filtered.

The filtrate was freed from excess of barium hydroxide with carbon dioxide, filtered and evaporated to small bulk, and again filtered. It was then found to reduce Fehling's solution strongly on boiling and to give the orcine and phloroglucine reactions, showing conclusively that a reducing body of some kind had been split off by the action of the sulphuric acid.

The barium precipitate from the above was shaken up with a small quantity of 0.5 per ct. hydrochloric acid, filtered, and the filtrate precipitated by adding an equal volume of alcohol. After again precipitating in the same manner the substance was filtered, washed in dilute alcohol, alcohol and ether, and dried in vacuum over sulphuric acid. The substance weighed 0.45 grams. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0.2326 gm. substance gave 0.0525 gm. H_2O and 0.1208 gm. CO_2.

0.1859 gm. substance gave 0.1009 gm. $BaSO_4$ and 0.0804 gm. $Mg_2\ P_2\ O_7$.

Found C 14.16, H 2.52, P 12.05, Ba 31.94 per ct.

Calculated for $C_{25}\ H_{55}\ O_{54}\ P_9\ Ba_5$=2184.

C 13.73, H 2.51, P 12.76, Ba 31.44 per ct.

This substance is therefore identical with the barium salt prepared from the previously isolated crude compound.

We are planning to carry out a complete investigation concerning this organic-phosphoric acid of wheat bran and its cleavage products. It is desired especially to isolate and identify the reducing bodies formed on cleavage with dilute acid. We also wish to take up the study of the nitrogen-containing substance and beg to reserve the further investigation of these bodies.

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New York Agricultural Experiment Station.

GENEVA, N. Y.

CROWN-ROT OF FRUIT TREES: FIELD STUDIES.

J. G. GROSSENBACHER.

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TECHNICAL BULLETIN NO. 23.

CROWN-ROT OF FRUIT TREES: FIELD STUDIES.

J. G. GROSSENBACHER.

SUMMARY.

Field studies from 1909 to 1912 have shown that in winter the bark on the trunks of fruit and other trees is often loosened or injured, near or below the surface of the ground; and observations made at intervals throughout the year show that the most severely affected portions of bark die and decay during the subsequent vegetative season, thus giving rise to crown-rot. It was also found that similar bark injuries, resulting in cankered areas or the death of the affected bark, occur in the bark of trunks some distance above ground — at crotches and on parts of branches which appear to be most subject to bending or strain in time of strong winds.

Neither the factors which induce the initial bark-injury nor those causing the subsequent death and decay have been determined experimentally, but were selected from the environment of affected trees in widely separated localities chiefly by elimination and deduction; however, the amount of accumulated circumstantial evidence is so large that there seems to be no doubt about the main causes involved and the time during which they are operative. From the data at hand it appears that the factors which are implicated in the production of the initial injuries are:

1st. An unusually large increase in the diameter of tree trunks during a vegetative season, necessitating an enormous increase in the area of the bark and resulting in high bark tension toward the end of the growing period, or the premature cessation in the process of differentiation of bark tissues owing to some adverse condition of the environment;

2nd. Low temperature and the resulting contraction of the bark with a consequent increase of bark tension; and

3rd. A wind-exposed location so that the bark at the crown or other places of bending of tree trunks or branches is subjected to great strain and consequent excessive evaporation during strong winds.

Such conditions result in loosening or injuring patches of bark, especially on the windward side of trunks and branches of trees which enter the dormant season with incompleted bark-growth or with high bark-tension in regions of bending and strain.

The death of injured or loosened patches of bark seems to be due to isolation and drying out, and it is often immediately followed by further disorganization and decay caused by the entrance of bark fungi like Sphæropsis and Cytospora. The wood underlying

winter-injured bark frequently becomes stained by the diffusion into it of some substance apparently originating in the disintegrating protoplasm of the affected tissues after resumption of vegetative activities in early spring, thus giving rise to "black-heart." Often the stained wood is then invaded by hymenomycetous fungi which decay the discolored parts and sometimes portions of unstained wood, thus resulting in "heart-rot."

The different varieties of apple trees often evidenced a marked variation in their relative susceptibility to the initial injury, but once injured they were equally subject to the rotting of the dying parts.

Crown-rot entails a heavy loss among most fruit growers in wind-exposed regions, and whether or not the initial injuries can be avoided remains to be determined. It seems possible, however, that their numbers may be reduced by inducing early and moderate growth in young trees, and by providing windbreaks or some device to keep the trees from swaying in the wind during winter. The observations also suggest that high-headed or severely pruned trees are more susceptible to the initial injuries. If young trees were carefully inspected in spring for evidences of loosened or cleft areas of bark most of the secondary injuries and rots could be prevented by carefully cutting away, at right angles to the surface, all injured bark and applying grafting wax or some good tar paint to the exposed wood.

INTRODUCTION.

Since the publication of a critical summary of the most important available papers dealing with crown-rot[1] and some related subjects most of the time has been devoted to a study of the disease. The field observations made plainly indicate that winter-injury is the first cause of the trouble; and also point to some of the predisposing and environmental conditions resulting in crown-rot. But a histological study of the initial injuries and their further development during the early part of the following vegetative season was deemed necessary for a better understanding of the disease. The field studies are discussed in the present paper while the histological work will be the basis of a subsequent report.

The observations were continued in 1910, 1911, and 1912. Not only the orchards in which the work was started but many others were studied with considerable care, especially during 1910 and 1911. The disease was found to vary much in different parts of the State and also in different orchards of the same locality, but in general it was much like that described in the above preliminary paper and like that found and described by many other investigators.

In these field studies an endeavor was made to find what environmental conditions prevailed in and about affected orchards and which

[1] Crown-rot, arsenical poisoning and winter-injury. N. Y. Agrl. Expt. Sta. Tech. Bul. 12:367–411. 1909.

varieties are most subject to the disease. It appeared that certain surroundings and locations are typical of crown-rotted trees, and other things being equal, that the different varieties seemed to possess a rather definite relative liability to the disease. An environment or season which induces the origin or production of tissues that for one reason or another could not be fully matured or differentiated before the close of the vegetative season, seemed to be associated with the occurrence of crown-rot. Whether the completion of growth or full maturation of the tissues is prevented by low temperature or some other untoward conditions such as drought, etc., the net result may be the same. A tree is thus forced into a dormant period of severe environment in an abnormal and susceptible state. The main varietal characteristics that were noticed as distinguishing the more susceptible from other varieties are the habits of rapid growth and early bearing.

Another environmental factor, which in general is noticeably related to the occurrence of crown-rot, is the wind. The relative wind exposure was frequently the only observable difference in the environment of neighboring orchards, one of which was severely affected, the other but little if at all. The direction of the prevailing wind of a locality or its direction during a critical period usually corresponds to the side of maximal injury.

The economic relations of crown-rot are of more consequence than appears at first sight, because the lives of affected trees are involved. In case of diseases of foliage or fruit the damages are largely confined to one season while from this type of disease a loss may be sustained which amounts to several years of time and a considerable outlay of money.

FIELD OBSERVATIONS.

INTRODUCTORY.

During late summer of 1909 a short study was made of a few crown-rotted orchards. The most severely affected trees were easily discerned at a distance by their rather sparse and prematurely yellowing foliage. On examination at close range it was found that such trees had large areas of dead, decaying bark somewhere on the stem, usually at the crown or about the bases of the upper roots. In fact in many such cases the decaying bark completely girdled the crown and in others the bark of all the lateral roots, as well as of the stump, was entirely dead and decayed, thus leaving only a few small roots under the center of the tree intact and available for the absorption of soil solutions. By making numerous cross and longitudinal sections it was found that in many instances both the alburnum and duramen were discolored or even decayed. In these same orchards, however, were apparently normal trees having injuries of the same type sur-

rounded by healthy rolls of callus. But on counting the annual growths in section it became evident that the injuries resulting in the exposure of patches of dead wood on normal looking trees occurred at the same time as those on the dying trees. The difference in the condition of the trees seemed therefore chiefly a matter of difference in the degree or extent of the initial injury.

In the orchards studied in 1909 no affected trees were found which had been injured just preceding that vegetative season; in most cases the initial injury had occurred two or more winters earlier. The assumption that crown-rot is primarily due to winter-injury had therefore not been deduced from observations on recent injuries but rather from the fact that the initial injuries had all occurred *between* the vegetative seasons, and could not on that account be due to mechanical injuries arising during cultivation or to other agents operating in the *growing season*; and also on the wide expanse and often general distribution between two particular seasons' growth, of a discoloration which frequently shows as a complete circle in cross-sections made even at some distance from visible external injuries of a tree trunk. It was during the summer of 1910 while studying a crown-rotted orchard near Coxsackie, N. Y., which had been briefly reported on before, that some early stages of the disease were observed. After examining the bark on the trunks of many normal looking trees, two of the Baldwin variety, which had been set nine years, were found to have complete girdles of dead bark extending downward about 8 to 14 cm. from the surface of the ground, and including the bases of the lateral roots. The wood appeared normal except for the discoloration of the outermost 1 to 3 mm. of the alburnum. Most of the dead bark was loose and slightly decayed, and the usual discolored zone just under the spring's growth extended some distance above any external indication of injury.

OBSERVATIONS DURING 1910.

The main object of the work in 1910 was to find all possible variations in the characters of the disease as it occurs in this State, and at the same time make special note of the environmental conditions, such as type and topography of soil, wind exposure and cultural history of affected orchards. In most cases the varieties and the relative degree they were affected were also recorded. The observations were made during late summer and fall.

Several orchards were studied around Geneva, Junius, Clyde, and along the west shore of Seneca lake; in and around Rochester, Coldwater, Sodus and Oswego; between Seneca and Cayuga lakes, around Branchport on Keuka lake, and about Highland, Milton and Coxsackie in the lower Hudson River valley. Most of the orchards were located by visiting fruit growers who wrote to the Station for information regarding their diseased trees.

The affected orchards near Sodus and those near Coxsackie, in which the work on crown-rot was begun in late summer of 1909, were visited but once in 1910. No additional trees were found affected in the Sodus orchard, but several of the remaining diseased ones had died. In the Coxsackie orchard only the two additional cases described above were found though others may have occurred, since not all of the trees were carefully examined below the surface of the ground. Twenty more of the trees in this orchard, discussed in the previous paper[1] on the subject, were taken out in 1910 and about twenty in 1911. The orchards around Geneva were under more continuous observation during 1910, but few new facts of any significance were obtained from them.

Some Branchport orchards.— The region about Branchport on Keuka lake is principally a grape-growing locality, although many small orchards may be seen there. About 3 miles west of the village is a small orchard of about 300 trees (about 10 acres) which had been set 9 years, 150 Ben Davis and the others Baldwin. The location is uncommonly high and wind-exposed. The soil is rather gravelly; though it seems fertile, for the trees look thrifty. The orchard had been tilled and cropped every year.

Considerable numbers of the trees were more or less severely crown-rotted or dead, the two varieties being apparently about equally affected. Some of the dead trees had been replaced by new ones. They seem to have been injured during the winter of 1908–09, *i. e.*, after they had been set 5 years. Somewhere between 60 and 70 trees had been injured at the same time; and although the more injured ones had thinner rolls of callus, they had the same number of annual growth additions since the occurrence of the injury as were present in the thicker rolls on the less injured trees. This is an unusually high percentage of injured trees.

The dead or decayed bark generally extended only about 2 to 4 cm. above the surface of the ground, and quite often as much as 5 to 6 cm. below ground. Only in the most severe cases were the upper lateral roots decayed, yet most of the injured trees were rather loose and more easily swayed by hand than normal ones. The trees were headed or pruned up rather high.

Twenty-one or more dead and dying trees were taken out in 1911. All of the less injured ones looked practically normal at that time, though small patches of bare wood, surrounded by thick rolls of callus, still gave evidence of the former injury. No additional cases of injury were found in 1911.

About 2 miles south of the above orchard is another small one of some 200 apple trees which had been set 18 years. It consisted of 50 trees each of Baldwin, Ben Davis, and Greening, 25 King and 12 each of Northern Spy and Spitzenburg. The orchard had usually been cultivated and often cropped. It is located nearer the lake and

[1] *L. c.*, p. 391.

has a few acres of forest about 300 m. to the northwest which may give some protection from severe winds in winter. The soil is deeper and less gravelly than in the first orchard described. Only Ben Davis, King, and Spitzenburg had crown-rot. Twelve of the Ben Davis, or 24 per ct., either died or had been more or less severely affected by the disease. No very evident difference could be found between the rigidity or resistance to swaying of the crown-rotted and uninjured trees. Even severely injured trees bore nearly normal crops during 1910, although a few of them died in 1911. No new cases developed in 1911. The rot in this case, as in the previous one, was also just above the ground line. All told, about 6 per ct. of the trees in this orchard were affected with crown-rot.

The Junius orchard.— Not far from Junius, a railway station a few miles north of Geneva, is another ten-acre apple orchard (300 trees, mainly Greening, Baldwin and Northern Spy) which had been set about 15 years. The soil is a sandy loam, lying very high and wind-exposed. The orchard had been cultivated and cropped until 1909.

The rot is often wholly above ground and somewhat canker-like, but in some cases it is just at or below the surface of the soil. Several of the tree trunks have the so-called "sun-scald" injuries on the west or northwest sides, to such an extent as to result in numerous irregular areas where the bare wood is exposed. The crown-rot injuries, when confined to small areas, are frequently also localized on the west and east sides. But it appears that the *distribution of the larger lateral roots has something to do with the localization of the injuries*, as noted in the paper previously cited.[1] The injury inducing the rot on these trees seems to have occurred during the winter of 1908–09.

Nine of the crown-rotted trees, including both Northern Spy and Greening, were either dead or will die soon; while most of the other 25 diseased ones seem to be recovering and will probably survive several seasons. The orchard is growing well and yielding good crops.

The Barnes orchards.— Two orchards were studied on the west side of the southern end of Seneca lake: one had been set about 12 and the other about 20 years. The trees were mostly Baldwin, Ben Davis and Greening, with a less number of Northern Spy. The soil is a rather thin, clay loam, with good drainage. There is no evident protection from the western winter winds. The orchards were plowed once a year and cropped every season.

About 4 per ct. of the trees in both orchards were crown-rotted, but the affected ones among the Ben Davis and Baldwin were more numerous than among Northern Spy and Greening. The initial injury seemed to have occurred during the winter of 1906–07. Most of the rot was several centimeters above the ground, although on a Northern Spy tree an almost complete girdle of exposed, dead wood was found just below the surface of the ground. In another instance

[1] *L. c.*, pp. 390–91.

the bark on all of the upper portion of an old callus, as well as that extending several centimeters above it, was discolored, and had some very small longitudinal clefts from which exuded a discolored "sap." The wood underneath the bark was also blackened to the depth of several millimeters. It could not be definitely determined whether the clefts had resulted from drying of the dead bark or whether they occurred before the bark died. On a portion of the dead bark the periderm was raised to form several blisters which also contained discolored "sap." The bark was not dead throughout its discolored portion, especially toward the upper and lateral limits of the newly injured area; in some places only the outer tissues of it were involved while the inner bark was at least partially living. It looked a little like a case found much later and shown in figure B on Plate XI. In the younger orchard many of the Baldwin and Ben Davis trees were affected with an irregular scaling off of the periderm on the southwest side of the stems from 5 to 15 cm. above the ground up to the lower branches. The scaling surface was very rough and irregular, and by scraping it with a knife was found to have small groups of dead, brown spots scattered thickly over it and extending almost to the wood.

The Coldwater orchard.— In a medium sized 20-year-old orchard near Coldwater were perhaps a dozen trees having more or less rot at the crown. In this case several Northern Spy trees were practically dead as a result of crown-rot. The land is almost level but the winter winds have a full sweep without obstruction. The soil is very sandy. It was cultivated until recently, but is in sod now.

The rot was almost wholly at or below the surface of the ground, and in several instances only involved the outer bark. In other cases a complete girdle of outer bark sloughed off just below the surface of the ground, leaving a somewhat irregularly pitted or pock-marked surface on the inner bark with a rather uneven cover of regenerated periderm. Two such corroded trees were found on which unevenness of the outer surface of the exposed inner bark was so marked that many of the pits extended to the wood. It seems as though scattered groups of cells in the outer bark had died and on account of their being so close together had become confluent by the dying of living groups between them. In some respects it resembles the distribution of groups of dead cells in cases of mild forms of so-called "sun-scald," above ground, except that in case of the underground injury the abundance of moisture favors a rapid decay of dead groups, and is more favorable to the formation of wound-cork over the outer limit of living tissues.

Maples around Geneva.— During the summer of 1910 many maple trees were found in the parks and streets of Geneva that had some loose bark. Plates XIV to XVII give an idea of the different types; including cases in which long radial clefts accompanied the loosening,

and some where there was apparently only a separation somewhere in the inner phloem with a subsequent dying of the loosened bark.

The most conspicuously affected trees seemed to vary in age from 9 to 15 years, and range from 11 to 18 cm. in diameter. In a small park near the Experiment Station were 12 *Acer platanoides* and 2 *A. saccharinum* trees injured to such an extent as to cause their removal in the fall of 1910.

In these particular cases the injury had occurred during the winter of 1909–10, as evidenced by the fact, shown in cross-sections, that the separation took place *after the close* of the growing season of 1909 and before growth was resumed in 1910. The portion of the tree trunk from 6 to 20 cm. above the ground nearly up to or even including the lower branches, was usually involved. In some instances the clefts in the bark were also accompanied by clefts in the wood, as shown in section in Plate XIV. Such clefts in the wood sometimes even extended entirely *through* the cylinder though in most instances they only went to the center or pith. All of the cases of bark loosening in and around Geneva dating back to the winter of 1909–10 were most severe on, and sometimes entirely confined to, the west or southwest sides. Usually there was sufficient injury in the inner bark of the entire circumference of the affected portion to show as a discolored circle in cross-section. On sawing short cross-sections from severely affected portions of a trunk the past season's growth of wood sometimes fell away from the central part of the section, along the discolored line. Usually both surfaces were of a dark brown color, but they were always more or less thickly covered by scattered pin-head-like, white specks. The raised white specks of one surface were found to coincide with those on the other when the pieces were placed together again. Apparently these irregularly scattered white specks were column-like living bridges connecting the wood of 1909 and 1910 through the thin, discolored or dead tissues between the two years' growths. The line of separation was chiefly somewhere in the phloem or the inner bark; but there was evidently much variation in the location of the place of separation, as judged by the differences in the growth and regeneration taking place in 1910.

Some of these matters will become clearer by a study of a cross-section of one of the tree trunks. Plate XIV is a section of *Acer platanoides*, taken about 25 cm. above the ground; its shortest diameter is 12 cm. In this case the wood cylinder was cleft into two nearly equal parts near the base, but about a meter higher up the cleft extended only to the pith; and towards the upper limits of the externally visible injury the wood was not cleft at all, although the place of injury is marked by a discolored circle nearly around the whole circumference. Plate XVI, figure A, shows a large piece of the same trunk. On the unpeeled part the bark, although having an unbroken outer surface, is undulating or wavy owing to differences in the rate

of growth at different places. These regions of different growth rate vary from 1 to 3 dm. in length and usually of less width, and are irregularly distributed over the surface of such injured trunks. Many maple-tree trunks may be found most anywhere having such irregularities of surface. Figure B on Plate XXI is a rather extreme case while that in figure A is very common. Plate XV is a section of the same tree taken 10 cm. above that of Plate XIV or at point 4 on Plate XVI. In comparing these two sections which are only 10 cm. apart, a striking difference is evident, giving some idea of how the unevenness of bark surface may come about. The regions of excessive thickening in this instance are seen to be due to a sort of double regeneration or growth. As may be seen from Plate XIV, the injury occurred when the outermost bark, 5, was still attached to the wood surface at 2; as may also be seen by the line of discolored tissue which makes a complete circle with the exposed wood surface. All the growth between 2 and 5 occurred, then, during the summer of 1910. It is evident, too, that in this case the bark and the wood were separated sufficiently by the initial injury to induce the formation of wood tissue and bark on the inner side of the old bark as well as on the outer side of the wood cylinder. At points 1 on this plate the regeneration along the inner side of the old bark failed, resulting in gaps covered by dead bark. In the section taken 10 cm. above this, as shown in Plate XV, none of the old bark on the left of the figure regenerated, but died. It, however, afforded sufficient protection to the regenerating wood surface to permit that to grow and develop a new bark under the dead one. It seems that the drying effects of the air prevented regeneration on the peeled side. About 15 cm. farther up the trunk, on the side retaining the bark, was a patch about the size of a man's hand where there was a total lack of regeneration of both the wood and bark, thus leaving a much depressed dead area surrounded by living bark. So this gives at least three degrees of difference in the subsequent growth of such a winter-injured tree: places of excessive thickening where a double regeneration occurs, others where the normal rate prevails, and yet others where no growth takes place but where the bark outside the injury dies.

It seems remarkable that "heart-rot" should have begun so soon after the initial injury: apparently nearly a fourth of the *old* wood had been permeated by the mycelium of a fungus. On putting a piece of the wood in a moist chamber an abortive fructification of a Polyporus developed on it in a few weeks. A Cytospora was fruiting all over the patches of dead bark shown on the left of Plate XV and also on the dead edges of bark shown in figure A of Plate XV. It is unlikely that the mycelium in the wood belongs to the same fungus as that fruiting on the bark.

The histological studies of such injuries, beginning before growth starts in the spring, and followed far enough into the summer to get

all the stages of regeneration as well as of the dying of the most severely injured bark, will make these matters clearer, and show in more detail how dead or canker-like areas like those shown in figure B on Plate V and figure C of Plate XII, develop on many fruit and shade trees.

Quite often the injury on shade trees is also chiefly localized at the crown, as it most frequently is on crown-rotted fruit trees. Numerous street trees in this city, especially maples along windy streets and those at windy street corners, have decayed areas of bark or exposed dead wood at the crown, like the ones shown in Plates XVIII and XIX. The trees shown in figure C of Plate XIX and figure A of Plate XXI failed to leaf out in 1912. Both also had large dead places just opposite the ones visible on the plates. Sometimes the injury occurs at the main crotches instead of at the ground or on stems between roots and branches, as shown on Plate XX, which is of a maple on an east and west street and has an injury on the east side. But in cases of this kind the side to be injured seems usually to be determined by the method of branching, just as the distribution of the upper roots seems to determine the place of injury when it occurs in the region of the root-crotches or crowns, as noted above regarding the localization of crown-rot of fruit trees. But, as will be seen later, when bark injuries result on that portion of the stems between the roots and branches they more constantly occur on the same side of all trees in a given locality, and without regard to the distribution of roots and branches. Usually trees along streets running east and west are more often injured. When both sides of the street are closely built up the injury is nearly always on the west side of the trees, but in very severe cases on the opposite side also. On streets running north and south which are rather windy, the injured places are on the south and north sides of the trees when both sides of the street are built up, but usually on the southwest side when the west side of the street is not built up. The injured trees in the east and west streets are more numerous than in the north and south ones. In other words, it seems as though the severity and direction of the winds have some relation to the occurrence of the injury.

The park referred to above, in which so many maples were injured during the winter of 1909–10, is located on a height of land at the northwest edge of the city and is notorious for its severe west and southwest winter winds. The trees were firmly rooted in heavy deep soil, and were far enough apart to have sufficient room for rapid growth. From cross-sections it is readily seen that growth had been very good.

A Trumansburg orchard.— A short distance northeast from Trumansburg, not far from the west shore of Cayuga lake, is a small apple orchard which is shielded from the north winds by a large peach orchard but has no protection on the west and south. The soil is a clay loam, with gravelly subsoil, and contains much humus.

The trees are chiefly Ben Davis, Hubbardston, and Northern Spy, which had been set 12 years and were uncommonly large for that age. They have had clean cultivation every year. Along a roadside fence on the east is a row of Ben Davis trees in sod that had been set 17 years, but which were not quite as large as those in the orchard set only 12 years.

On November 1, 1910, a dozen trees of the 12-year-old orchard had been taken out because they were practically dead from crown-rot; and 22 more were more or less severely affected, while the remaining 65 trees in the orchard were apparently uninjured. Most of the dead and injured ones were Ben Davis although a few Hubbardstons were also affected. Those dying in 1910 and the normal looking ones having crown-rot seemed to have been injured during the winter of 1908–09. The diseased trees and gaps were much more numerous in the two west-end rows, although they were scattered throughout the orchard. The Ben Davis trees were usually injured just above the ground, while two Hubbardstons were found having almost complete girdles of dead bark underground, around the bases of the uppermost lateral roots. The injured Hubbardstons did not stand very firm but the affected Ben Davis trees apparently stood as firm as the uninjured ones. Even at this late date (November 1) one could pick out some of the injured trees by the difference in the color of their foliage.

The rolls of callus around the old wounds were uncommonly thick on the less injured trees, while on those dying in 1910 the growth of the past season had been but little.

Here is another instance where the rapidity of growth seems to be related to the occurrence of the initial injury. Like some instances discussed in the above cited paper, trees growing slowly or in sod are apparently less subject to this disease. The row of Ben Davis trees in the sod along the fence, and growing so slow that the 12-year-old trees under cultivation were even larger, had not been affected. However, when judged by the production of fruit the cultivated trees were more profitable.

OBSERVATIONS DURING 1911.

Since the observations in 1910 resulted in broadening the concept crown-rot, and suggested several environmental conditions which appear to be implicated or to co-operate in the production of the disease, it seemed desirable to obtain more definite information during 1911 concerning the details and range of variation of the initial injuries.

During 1911 orchards were studied around Geneva, Medina, Weedsport, Clyde, Branchport, Middlesex, Hemlock, Milton, Glens Falls, Le Roy, Interlaken and Sodus.

Occasional night frosts usually do not defoliate the common deciduous trees of this region in autumn. In normal seasons vegetative activities of our trees cease about the latter part of October; yet apple trees may retain their foliage until the latter part of November, if the rainfall has been considerable during the months of August and September.

Göppert[1] gives the vegetative season or rather the time between the appearance and fall of the leaves of pear and apple as 184 days.

In general it may be said that the conditions were very good for vigorous and late growth in the fall of 1910. The rainfall in August and September were 138.9 mm. and 83.6 mm. respectively, while the averages[2] for those months from 1882 to 1910 are only 96.5 mm. and 55.1 mm. The temperature was also conducive to late fall growth. The averages in 1910 for September, October and November were 17.3° C., 12.7° C., and 2° C.; while the monthly averages of those months for the years 1882–1910 inclusive are 17° C., 10° C., and 3.7° C. respectively. It is thus seen that the temperature for September and October in 1910 was above the 29-year average for those months, and that the November temperature fell below the general average for that month. To sum up, then, the temperature and rainfall during August and September were above normal in 1910, and the temperature in November was below the normal. In fact, the general impression prevailed that wintery weather began about November 12 instead of the latter part of December and continued cold into the early part of January, 1911, when it unexpectedly thawed during terrific west and southwest winds which lasted several days.

It was during the latter part of this warm period in January that winter-injury was found to have occurred quite commonly on fruit trees in the western part of the State. The inner bark and very often parts of the cortex, medullary rays and pith of stems and ascending branches of young apple, cherry, peach, pear, plum, and other trees were found much discolored in spots. Some complaints and requests for advice from fruit growers also came to the Station regarding the discoloration evident on the west or southwest sides of trunks and branches of young pear and apple trees or on smooth-barked, upright branches of older pear trees.

Some Medina orchards.— While visiting some such winter-injured pear orchards near Medina, N. Y., on January 17, 1911, quite a number of young apple trees were found which had loose bark on the stems at the ground. The finding of one or more short and very narrow clefts in the bark of the crowns led to the discovery. The loose bark was living and normal in every way, except that there was a faint brownish discoloration along its inner side and slightly more along the radial cleft. The separation had apparently occurred in the phloem or just outside the cambial region in most cases; but two were found on which the separation had occurred in the cortex, so that

[1] Page 269 of his monograph cited on page 49.

[2] From meteorological records kept at this Station.

only the periderm with some adhering green cortical parenchyma was loose. The loose patches of bark were mostly on the west, northwest, and southwest sides, and their length or vertical extension usually exceeded that of the clefts by 3 to 10 cm., *i. e.*, it seemed that the formation of radial clefts in the bark need not necessarily accompany tangential ones or bark loosening.

These trees were of the Baldwin variety and had been set 2 years; the rows alternated with rows of Bartlett pears, set the same length of time. About 80 per ct. of the pear trees had become severely winter-injured. The soil is a sandy loam, lies rather high in a comparatively flat country with no wind protection on the west. The orchard had been cultivated and cropped to cabbages, tomatoes, etc.

Diagonally across the road to the northeast of this orchard is a smaller Bartlett pear orchard of the same age, condition and culture; but which has a dense old apple orchard on its east side. The bark of quite a number of the pear tree trunks and larger branches was blackened on the west side. But only about 20 per ct. of them were severely injured. Along the east side of the above old apple orchard is another two-year-old Bartlett pear orchard under the same cultural and soil conditions but having only about 1 to 2 per ct. of the trees severely winter-injured, and nearly all of those were in the south end of the orchard which extends some 50 meters farther south than the apple orchard, along the east side of a barn lot. The most severely injured trees were in the portion of this southern extension just east of the gap between the old apple orchard and the barn.

There seems to be no doubt but that the direction of very high winds at certain times during the winter has a relation to the production and therefore to the localization of this type of winter-injury as well as to the type resulting in crown-rot. In this case both types of injury probably occurred sometime between the first of November, 1910, and the middle of January, 1911; for at the time of the last field work in late October no such injuries were found about Geneva and a number of other places visited during the latter part of the month. The detailed discussion of these injuries can more profitably be taken up in connection with the histological studies of crown-rot.

On April 4 another visit was made to the Medina orchards to study the further development or changes that had taken place in the winter-injured trees. The clefts in the loose bark of the Baldwin trees mentioned above had become slightly longer and considerably wider. In two instances the bark had died over about one-half the loosened area, amounting to about a fourth of the circumference of the tree, but in most cases the loose bark was yet living to within about 2 mm. of the radial clefts and with but very little additional browning on its inner side. But the wood of the stems exposed by the clefts had become more browned. The fruit grower was advised to remove all the loose bark with a sharp knife by cutting it out at right angles to the surface, and then

covering the exposed wood with grafting wax. While removing the bark it was found that in cases where it had been in close contact with the wood, the latter was often stained dark on the surface of contact with the bark.

Two of the Bartlett pear trees along the road were also found to have patches of loose bark to the extent of about a third the circumference and 5 to 10 cm. high on the west side of the trunk, at the surface of the ground. One of the injured trees had a loose, tin sleeve about 35 cm. high, surrounding the basal end of its trunk as a protection from rodents. The bark had not only died about the narrow clefts, but nearly all of it that was loose had died and the wood underneath had become stained black to the depth of about a millimeter.

Some Geneva orchards.— About a mile west of Geneva is a small young apple orchard, set 5 years to alternate trees of the Bismarck and Baldwin varieties. The orchard is just south of an east and west road in a slight depression which drains to the north, and just east of a farmhouse and numerous other windbreaks. However, there is no windbreak on the northwest, or on the north side of the road where the land is lower. The orchard was cultivated every year and the trees had grown very rapidly. Conical mounds of soil, to the height of about 15 cm., had been heaped about the crown of the trees in the fall of 1910, and left there until the latter part of April, 1911.

When the orchard was examined on May 6, 1911, for winter-injury four trees (1 Baldwin and 3 Bismarck) were found having loose patches of bark on their stems just above the ground. Two of the Bismarck variety had only the periderm with some adhering cortical parenchyma loosened on the west side over an area extending about one-third round the trunk and 10 to 15 cm. high. On removal of the dead periderm the exposed cortical parenchyma had a very uneven, corky surface; and at the line of radial rupture of the periderm the bark had died practically to the wood. On a Baldwin and on a Bismarck tree some bark was loosened on the northwest and east sides, beginning above the ground and extending upward. The loosened patches were cleft longitudinally, nearly their entire length. The edges of the cleft bark stood several millimeters apart in the middle and were much browned. The wood exposed through the crack and that immediately under the edges of the bark was also brown and dead on the surface; but back under the loose bark it had a white glistening surface with a sprinkling of small brownish specks somewhat unequally distributed. The inner side of the bark had a similar appearance in the case of the Bismarck tree.

The above Baldwin tree with loose bark had a cleft about 6 cm. long on the northwest side and stood open about 4 mm. A crescent-shaped piece of the loose bark 5 cm. long and 2 cm. wide in the middle had died and become dry on the south side of the cleft, but along the

other side of the cleft it had died back only about 4 mm. from the cleft edge.

The Bismarck tree referred to above had a radial cleft in the loose bark on the northwest side 17 cm. long, and another 7 cm. long in a loose patch opposite the large one. The edges of the larger cleft stood apart 6 to 7 mm. in the middle, and the bark had died back about 6 to 10 mm. on both sides of the crack. Figure A on Plate I is a photograph of this tree trunk, taken May 7, 1911. Without moving the camera all the loose bark was removed and the tree rephotographed; but the negative was spoiled, and therefore another was made the next day. As may be seen from figure B the camera was a little closer, although an attempt was made to set the tripod legs into the same holes they had occupied when figure A was made. A number of very fine wind-checks are also noticeable on the exposed surface.

These figures show that the cleft did not extend to the ground, although it went several centimeters below the surface of the conical mound of soil which had been heaped about the tree during winter. The lower end of the shorter cleft, on the opposite side of the trunk, *began* at the same height as the lower end of this cleft. The longer cleft extended practically the entire length of the loose patch of bark. On May 7, the tree measured 23.5 cm. in circumference. After removing all the loose bark on both sides of the trunk only two narrow bands of adhering bark were left between them. That on the northeast side measured 3.75 cm. and the one on the southwest only 2 cm., thus leaving a quarter of the bark adhering to the wood as bridges.

The loose bark was alive except along the margin of the cleft, and seemed to have increased slightly in thickness, for its inner surface was greyish white and glistened like a regenerating surface. The exposed wood surface had a similar appearance except that an irregular, band-like region, exposed to the air by the crack in the bark, was brown; and that a few other brownish spots were scattered irregularly over the other portions of it. But, even though this wood surface was exposed to the air about 24 hours before being covered with grafting wax, considerable regeneration occurred later, as shown in figure A of the next plate, by the bursting of the wax cover (August 15). On September 11 the wax was carefully removed to determine the amount and appearance of the regeneration which had occurred. Figure C of Plate II shows that a narrow strip of wood surface exposed by the cleft as well as various other places failed to regenerate or did so only partially. At that time the tree measured 28 cm. in circumference. The bridging band of bark on the southwest side had increased to about 5.5 cm. in width by lateral growth, and that on the northeast side to about 8 to 9 cm., although its lateral limits were not easily made out on account of the almost perfect regeneration taking place on the adjoining surface of exposed wood.

Figure B on Plate II shows that the tree looked normal in every way. In fact it was impossible to tell any difference either in the growth, fruitfulness or color, between this and other trees of the same variety in the orchard.

Across a little ravine on the east of this orchard is another small apple orchard which had been set 7 years and cultivated regularly. About 6 out of each hundred trees were affected by crown-rot, as shown in figure B on Plate XII. Three of the trees died in 1911 and several had died the year before. The initial injury occurred during the winter of 1908–09, as indicated by cross-sections through the affected trees.

Sometimes radial clefts occur in the bark of apple and other trees when in reality it is loosened but little, or not at all. In the case shown in figure B on Plate XVII a cleft occurred during the winter of 1910–11 but no loosening of the bark had taken place. In such instances it appears as though the normal maximum bark tension occurring just before normal bark roughening or scaling begins is sufficiently increased by low temperature to result in abnormal ruptures.

The Weedsport orchards.— A short distance northwest of Weedsport is a 7-acre apple orchard 5.5 acres of which had been set to Baldwin trees in spring of 1909, with Ben Davis as fillers; *i. e.*, with trees of Baldwin and Ben Davis alternating in each row. The other 1.5 acres was set at the same time to a mixture of Northern Spy, Wealthy, and other varieties. The orchard is on high land draining toward the southwest, but has a rather dense low growth of forest on the west which also has a scattering extension on the north around the west end of the orchard, as shown in figures A and B on Plate VII. The south side, however, is wholly open and fully exposed to the south and southwest winds.

The whole orchard was thoroughly fertilized and cultivated without being cropped. The trees were banked with soil in the fall of 1910, which was removed in spring of 1911. The Baldwin trees had been *headed high* while the Ben Davis trees were pruned but little.

The first visit was made to the place May 18, 1911, when it was found that about 85 per ct. of the Baldwin trees had girdles of dead or dying bark about their trunks, usually beginning 6 to 10 cm. above the ground and extending upward 10 to 18 cm. Apparently, then, the injury occurred about the upper surface of the soil mounds as it did in the orchard near Geneva, discussed above. The clefts in the bark were usually on the southwest or south sides, but on account of the fact that the bark of many of the injured girdles had already died and become dry and wind-checked in various places, it was impossible always to distinguish between primary and secondary cracks. Unfortunately none of the dozen films exposed during this visit proved good enough for reproduction, but the injured places

looked much like those shown on Plate III with much of the regeneration left out.

In some cases, however, considerable growth and regeneration had occurred resulting in much unevenness in the bark; while in others large areas of bark were only partially loosened, having no radial clefts and still having a green color externally. Such loosened bark had a rusty brown color with a sprinkling of glistening white pinhead-like specks on the side which had been in contact with the wood. A closer study of the inner surfaces of such pieces of bark and of the wood surfaces from which they were taken plainly showed that the white specks of one corresponded exactly with those on the other. It looked as though the bark had not been entirely separated from the wood, but that here and there living groups of cells still maintained a connection between bark and wood. The injury and separation had occurred in the phloem region. In very many other cases carefully examined, such partially adhering areas of bark were nearly dead throughout and there was considerable brownish slime between the bark and wood with still an indication (at intervals) of glistening white specks indicating a living connection at a few points. But the wood had become discolored to the depth of 2 to 12 mm. under such areas.

In many instances, however, the whole of the bark had died, become wind-checked and parts of it stood out from the smooth wood surface. In general, on trees where the bark had been nearly or entirely loosened it had apparently been dead for some time, and there had been little or no staining of the wood beyond the outermost 1 to 3 mm.; while in the cases where the bark had been only partially separated from the wood it was usually yet partially alive and the wood underneath was stained more or less.

The most remarkable feature about this case is the fact that although receiving the same treatment in every way except in the matter of pruning, there is such a great difference here between the Ben Davis and the Baldwin varieties as regards their susceptibility to the injury. In all the orchards studied before, these varieties were not found so markedly different in their relative resistance, while in this case the Ben Davis trees were wholly immune and the Baldwins nearly all injured more or less. Since no difference could be found in the environment or cultivation of the trees it seems legitimate to infer that the above mentioned difference in the pruning of the two varieties may have had some relation to the occurrence of the injury; that is, the high-headed trees were injured while the low-headed ones remained normal.

The Northern Spy, Wealthy and other varieties referred to above had only a few trees among them slightly affected, and they were mostly of the Wealthy variety.

The fruit grower was advised to cut off, below the dead region, all the trees which were entirely or almost entirely girdled and cover

the cut end with grafting wax or paint; and then to select the best sprout for a tree and recut the projecting stub close to it to make the least possible bend in the resulting trunk. It was advised to cut out all loose or injured bark of trees having as much as a third of the circumference of intact bark and to paint the exposed wood to prevent the entrance of water and decay.

On August 4 the orchard had a different appearance. It seemed as though it were a most excellent orchard of Ben Davis with a few scrubby Baldwin trees and sprouts scattered through it. Figure A on Plate VII is taken down a diagonal row of Baldwins or where they should have been, and to the right and left the Ben Davis rows are seen to look thrifty. In the foreground is a Baldwin sprout, and farther down the row are two Baldwin trees with splashes of white paint covering some exposed wood on the south side. The injured Baldwin trees grew but little and their foliage was rather sparse. A closer view of an injured one is shown in figure C on Plate VI, which was taken on the above date. Figure B of the same plate shows another one which had given rise to a thrifty sprout, apparently due to the poor water conduction of the injured trunk. Most of the sprouts on the stumps had made a good growth, but a strong wind storm a few days before had broken off many of them. The appearance of the sprouts and the manner of cutting off the tree are shown in figure A of Plate VI.

At the time of the first visit to the above orchard on May 18, two other orchards near it were also examined for winter-injury. One of them, consisting of rather scattered trees which had been set 6 to 10 years, was just south of the above orchard and consists of various varieties. On a knoll a 10-year-old tree had small patches of loose bark on the south and north sides of the crown at the surface of the ground. The loose patch on the north had a cleft about 7 cm. long and was alive except at the cleft edges. By cutting out the loose bark it was seen that regeneration had occurred on its inner side near the cleft and that farther from the cleft were very numerous glistening points which corresponded with similar points on the wood surface. It appeared as though further growth might have turned out the edges of the bark at the cleft as it did on the maple trees discussed above and shown on Plates XIV and XVII. On the south side of the tree the cleft was only about 4 cm. long and the bark was lying rather firmly against the wood. It was not removed to determine the extent of loosening because it appeared as though it would be better for the tree to leave it. It seemed likely that in this case further growth would go on as it did on the one shown on Plate VI of the above cited paper on crown-rot.

The other orchard referred to above is about a half a mile farther south, set four years and in an exposed situation. The grower said that two years before a few of the trees had died in mid- and late-summer, and on examination were found to have wide girdles of

dead bark just above the ground. A number of the old wounds surrounded by rolls of callus were in evidence. A few of the trees were injured in the winter of 1910–11. In these instances, which were not very severe, the loose bark seemed to be dying out to the very limits of the injured areas.

Some Clyde orchards.— On the northwest edge of the village of Clyde are some apple orchards that were also injured in the winter of 1910–11. The most severely affected one consists of about 8 acres which had been set 4 years to Baldwin and Greening. The orchard lies east and west across the crest of a north and south hill. The east end of the orchard seems to be shielded from north and west winds by the crest of the hill and a house and trees respectively; but the middle and western portions are fully exposed to the wind. The soil is a light, clay loam, with a gravelly subsoil. It had been heaped about the trees over winter and removed in spring. The eastern part of the orchard has richer soil than the central and western part; and as a result the trees were appreciably larger in the eastern part.

On June 3, when the orchard was first visited, about 20 per ct. of the trees had more or less dying bark on the stems. Perhaps three-fourths of the injured ones were Baldwin. There were as many injured trees in the east half as in the west half of the orchard, although the former was less exposed to the wind.

Many of the affected trees were completely girdled and a few of them could be distinguished at some distance by a slight yellowish tint of their foliage. Those with a bridge of living bark as wide as one-eighth of the circumference looked normal in every other respect. When the injured or loosened area of bark on a tree consisted of not more than half the circumference it was usually confined to the southwest side of the trunk.

The injured places began from 3 to 8 cm. above ground and extended up the stem from 7 to 18 cm.; but most commonly about 11 cm. The dead or dying bark was usually cleft in a number of places, and in cases of complete girdling much irregular callus growth had developed on the trunk over areas where the loose bark was not cleft By turning to Plate III the matter is more readily understood. Figure A shows a typical example in which much regeneration had occurred on the wood surface where it is covered by the old, dead bark; while at the cleft no growth had resulted. In comparing it with figure B, from which the loose bark had been removed, the regenerated part is even more prominent. There were two narrower clefts on the other or northeast side of the trunk. The figures also show that the removal of all dead and dying bark included some above and below the clefts which was not actually loose, but which was discolored to a dark brown in the phloem region. The wood underneath the discolored and partially live bark had also become

stained to the depth of several millimeters. Another and more striking illustration of this is shown in figures C and D which are also of a tree trunk before and after removing the dead and dying bark. In this case the bark was dying as much as several decimeters above the externally visible injury and the wood underneath was much discolored, apparently by the diffusion into it of some oxidizing agent coming from the disorganizing phloem. Figure E shows an instance where the cleft on the southwest side was uncommonly long; *i. e.*, where it had apparently extended to the *full length* of the bark injury. As may be noticed, the area over which regeneration from the wood resulted *is correspondingly long.* Although this tree had a much longer girdle of cleft, loose bark than those shown in figures A and C, more regeneration resulted and practically no additional bark was found discolored in the phloem, and no staining of the wood occurred with the exception of the superficial layers. And what is more interesting, the foliage of this and other similarly injured trees was of normal color, while those with much *stained* wood could be detected by the yellowish tint of their foliage.

Another thing noticeable from these figures is that some regeneration had occurred over practically the whole wood surface where the bark was completely separated but yet remained as a protecting cover, while above and below the places of complete separation the bark was usually more or less discolored in the phloem region and the underlying wood stained. That is, actual separation of the bark did not appear to be as harmful to the tree as a certain amount of injury in the phloem *when the bark was left on.* But in cases where the tree was *not completely* girdled the disorganization in the phloem of partially loosened bark, was not so marked; and the staining of the underlying wood less extensive.

The orchardist was advised to cut off all girdled trees, remove all loose or injured bark from the less injured ones, and cover the exposed wood with grafting wax or paint. Figures A and C on Plate IV and figure A on Plate V show some of the typical results as they appeared August 3, 1911. Figure B on Plate IV is one of the sprouts grown on a Baldwin stump. Grafting wax had been used to cover the exposed wood. The wax seems to loosen from the margins of the wood and frequently crack open; no doubt some tar paint is better to cover *dead* wood surfaces, as may be seen from the results obtained in the above Weedsport orchard.

On a neighboring bleak clay hill is a very small mixed orchard of Ben Davis apples and peach trees, set 8 years. At the time of the first visit in June, 1911, a number of winter-injured Ben Davis trees were found in this orchard. There were perhaps 2 dozen apple trees in the orchard, very large and thrifty in appearance. The bases of the trunks had been incased in veneer protectors about 4.5 dm. high which were still on the trees. On removal of the protectors 5 of the trees were found to have areas of partially loose and decaying

bark on the northwest side beginning 5 to 8 cm. above the ground. But the injuries usually covered less than half of the circumference. In three of the cases unmistakable evidence of very narrow longitudinal clefts were found in the injured bark, but in the two others which were smaller, no indications of clefts could be seen. Figure A of Plate V shows one of them after it had been covered with wax.

In view of the numerous other observations of this type of injury on trees without the veneer it seems highly probable that the protectors had no causal relation to the initial injury in this instance.

Two other Branchport orchards.— About 1.5 miles southwest of Branchport is a small 9-year-old apple orchard which consisted of 130 trees of Baldwin, Stark and Northern Spy. The land slopes toward the east and the soil is clay. On the south side is a small patch of forest while the west and northwest are open.

The trees had been thoroughly fertilized and cultivated; they had made a rapid growth. In the spring of 1910 while looking for "grubs" the grower found the bark of some trees injured at the ground, and in 1911 he found many with decayed bark at the crowns. On May 11, 1911, 8 of the trees had been taken out and perhaps 15 more were considerably crown-rotted. Their general appearance was like that of figure B on Plate XII.

Trees of all three varieties were affected but the injured ones were principally of the Baldwin variety.

Adjoining this orchard on the north was another with nearly twice as many trees, and of the same varieties, but apparently without a single tree injured. These trees were not as large as those in the neighboring orchard although they had also been set 9 years. The soil and slope of land were identical and the wind exposure seemed even greater. The trees were also headed about alike in both orchards. The only difference that could be found in the two was that the crown-rotted one had been thoroughly fertilized and cultivated while the other remained in sod.

A Middlesex orchard.— About a mile southwest of the village of Middlesex is a Baldwin apple orchard of 150 trees on high land, and with rather thin, gravelly soil. It is fully wind exposed in all directions.

The trees had been set three years and the orchard had been cultivated and cropped. The trees seemed rather small for that age. On June 2, six of the trees were found to have injured bark on the northwest side, near the ground. The most severely injured one had a patch of partially loose, cleft bark on its northwest side which extended more than half way round the trunk. Six injured trees had been replaced by the grower, in May, because they seemed to be dying.

A Milton orchard.— During the second week in June in a clean cultivated orchard about 2 miles west of Milton 3 Baldwin trees were found having small areas of loose, cleft bark at their crowns. About

half the loose bark was entirely dead and the remainder was evidently dying back to the *limits* of the loosened areas where a rim of callus had already begun to form.

The Hemlock orchards.— Near the eastern shore of Hemlock lake on a high, clay ridge is a small apple orchard which had been set 5 years. The orchard had been thoroughly cultivated and fertilized without being cropped, and the trees were large and thrifty looking for that age.

About 7 per ct. of them were injured at the surface of the ground. But only on four was the bark loosened in the phloem region, when the orchard was visited on June 5. A half dozen had large patches of periderm with some adhering cortical parenchyma cleft and loosened on the northwest side. The areas of bark loosened in the phloem region extended less than half way round the trunks, and only half the injured bark was dead. In spots where it was wholly separated from the wood considerable regeneration or callus formation, of the type shown on Plate III, had occurred on the wood surface. On two of them there was some regeneration on the inner side of the loose bark somewhat like that shown on maples in Plate XIV, but only in small patches. But on one of the trees most of the callused bark was evidently dying. It appeared as though the wound might become a ragged one in the course of a year or so more, like the old wound shown in figure A on Plate XIII. An irregular rusty looking surface had developed on the exposed patches on trees with the periderm loosened. The covering seemed to be made up of dead cortical tissues and newly formed cork.

In another small apple orchard on the same clay ridge and about a mile south of the one described above, there were 5 trees with yellowish foliage. They had apparently been set 8 years, and the orchard had been cultivated and cropped. The diseased trees were found to have crown-rot resulting from injuries which occurred during the winter of 1909–10. They were all completely girdled at the surface of the ground, and nearly all the dead bark had decayed, leaving a smooth surface of dead wood exposed much like that shown in figure B on Plate XII, except that the girdles were only about one-eighth as wide.

Some Glens Falls orchards.— In a wind-swept region a few miles north of Glens Falls are a number of small bearing apple orchards which were injured during the winter of 1910–11, but only two of them were actually studied. One orchard which consisted of perhaps two dozen trees of various ages and varieties adjoins farm buildings on the west and had 5 or 6 injured trees, chiefly 10 or more years old. The soil seemed productive and was under cultivation.

Two of the affected trees were crown-rotted in narrow bands extending about half way round the trunk at the surface of the ground. The initial injury on these seemed to have occurred during the winter of 1909–10. The other affected trees were injured in the winter of

1910–11, and their general appearance was normal. But the bark was cleft and loosened on the north side as may be seen from figures A and B on Plate VIII. The clefts did not extend the entire length of the loosened area; for example, in the case shown in figure A the bark was loosened or nearly so from a few centimeters above ground up about twice the length of the cleft and nearly half way round the trunk. About two-thirds of the loosened bark was still alive but in a wilted condition. The portions around the clefts were dead and dry. No definite regeneration had occurred between the bark and the wood but considerable disorganized brown slime was present under it.

Figure B shows a case where the bark was loosened higher up on the trunk and where less than the circumference was involved. In this instance the loosened bark was nearly all alive and the wood, except that immediately under the cleft, was discolored but little and seemed to be regenerating and apparently establishing new connections with the live bark in many places. No indications of corky layers were noticed between the bark and wood, as was the case in maples shown on Plates XIV and XVII. Otherwise these cases were very similar to those so frequently found on maples. The nature of the injury and the type of regeneration seen in figure B of Plate VIII seemed more like that shown on Plate VI of the above cited paper on crown-rot, and like that shown in figure B of Plate XVI of the present paper.

The other orchard studied is about 100 meters northeast of the first one; it is in sod and seemed to have a more sheltered location. The trees were mainly from 9 to 10 years old. The grower said that they had been pruned very lightly until June, 1910, and January, 1911, when they were severely pruned. The soil was very compact but apparently productive, for the grass and trees looked thrifty. The trees were strongly rooted and stood firm.

The orchard consisted of 89 trees of Baldwin, Northern Spy and other varieties. Thirteen trees were completely girdled and 28 others were less severely injured. The bark was cleft more or less on all affected ones, but usually only a fraction of the length of the loosened areas. Most of it was also partly alive, at least the part somewhat removed from the clefts. The height of the girdles of loosened bark varied much on different trees and sometimes they were much higher on one side than on the other, as shown in figure A on Plate IX. Those trees having only a slight injury most commonly had that on the northwest side near the ground. But it was usually impossible to say which side was most affected in case a tree was completely girdled, unless the girdle was high on one side. Figures A and B on Plate IX are typical examples of complete girdling.

Figure A is an instance in which the greatest injury had occurred on the northwest side, extending from the ground up to the branches; while on the opposite side it extended only a few decimeters above the ground. There was a cleft in the bark on the northwest extending

about half or two-thirds of the way up to the branches, and a few very short clefts or perhaps wind-checks were scattered over the base of the south side. About three-fourths of the loosened bark was partly alive, and in some spots more or less regeneration had occurred on the wood surface under it. At the right of figure A on Plate IX some such spots are readily seen as darkened areas. Most of the bark on the northwest side, including the piece hanging by its upper end, was dead and the wood surface underneath had a dark brown color. The loose bark taken from B had been cleft at a few places near the ground, but none of the clefts reached more than a fourth of the way up the trunk. All of the bark except that surrounding the clefts still appeared normal externally but on removal was found to have a rusty or dark brown color on its inner side. Between the closely appressed bark and the wood was some disorganized and discolored slime. The loose bark was not uniform throughout; there were often large areas in which it was dead and discolored only to the outer layers of cortex, while in others even the whole of the cortex had died in small spots. It seemed evident that the entire loosened portion of bark was dying from within outward.

For trees of that age this is an unusually high percentage of injured ones. Over 46 per ct. of them were more or less severely affected and over 15 per ct. were completely girdled or fatally injured. Yet, when the orchard was first seen the foliage of all trees appeared of the same normal color, although after the loosened bark had been removed one could readily imagine that the leaves of most of the girdled trees were of a yellowish or lighter shade of green than the others. That seemed most noticeable on trees like that in figure B on Plate IX, which had much discoloration of the wood.

Several of the less severely affected trees also had injured bark in their lower crotches. In some cases the injured bark looked discolored or dead while in others it still appeared normal externally although on removal was found to be partially separated from the wood and to have a rusty to brown dark phloem. In other instances small clefts or wind-checks were present in the dead bark, but most frequently it was uncleft. When patches of injured bark had completely died they looked like typical crotch cankers. The dead areas were sunken below the level of the normal bark and were unusually delimited by a more or less conspicuous line of fissure. In cases where the cortical portion of the affected bark was yet mostly alive the fissure was less conspicuous or even entirely absent, but on cutting out a piece across the indefinite region of transition from the injured to the normal bark the cause and location of the cleavage became apparent. A definite but thin ridge of callus was found running around the periphery of the injured areas, indicating that the cause of the fissure is the increase in thickness under the normal bark and its absence of thickening, combined with the drying out and dying of the bark, over the several affected areas.

Sphæropsis and Cytospora were found sporulating on much of the loosened bark which had died, and occasionally Sphæropsis was also present on bark that was loosened but not dead throughout. In a few instances Sphæropsis was fruiting on dead patches of bark about injured crotches. It appeared very probable, however, that the fungi were only saprophytes in these cases because the bark was not found injured beyond the first callus that had formed in early summer under the loosened bark around the periphery of the initially injured regions.

The Interlaken orchards.— Along the western edge of Interlaken is a small apple orchard which had been set two years. The land has a gentle slope toward the north and is fully wind exposed on the north, west, and southwest. It had been thoroughly cultivated.

There were 60 trees of each of the following varieties: Hubbardston, McIntosh, Rome and Wealthy. When the orchard was visited on August 5 the leaves on all but three trees had a normal color. On these the foliage had a yellowish tint. The lower portion of the stem of one of them is shown in figure C on Plate X. The three trees with yellowish leaves were completely girdled as shown in this figure, and apparently the injured region of bark was not wholly separated from the wood for it was still adhering in many places although it was much dried and wind-checked. A very thick roll of callus had formed along the upper edge of the girdle but no regeneration occurred on the wood of the injured region. The wood was much discolored to the depth of over a centimeter. A fairly vigorous sprout had developed from the stump of every girdled tree.

Most of the other affected trees had injured areas often extending as far as half way round the trunks, as shown in figure A of the above plate, which was taken after removing the dry bark that was still partially adhering to the dead wood underneath. None of the affected trees showed regeneration except along the edges of live bark as shown in A.

The Hubbardston trees were apparently more susceptible than the other varieties. The three discussed above were of that variety, and 20 others were less severely injured. Only 2 per ct. of each of the other varieties were affected.

About 2 miles farther northwest and not far from Lodi is an orchard of about 17 acres which had also been set 2 years. The soil was richer and the trees were larger than in the above orchard. It had also been cultivated and cropped. On the south side is a dense old apple orchard but otherwise the exposure was almost the same as that of the one discussed above.

It consisted of 200 trees each of Baldwin, McIntosh and Wealthy; 175 Greenings, 100 Hubbardstons, and a few trees of some other varieties. About 40 Hubbardston trees were injured on the northeast side, but in most cases the affected areas covered less than half the circumference of the stems just above the ground. On a few

of them there was a narrowing upward extension of the injury as shown in figure B on Plate X. In this instance the grower had previously sent a man to clean out the wounds and paint them, but as may be seen from this figure the injured bark had not all been removed before paint was applied; at the upper and lower ends the paint had been applied to the affected bark instead. The photograph was taken after the additional dead bark was removed, thus showing the result of careless work.

A few McIntosh, two Northern Spy and about 15 Baldwin trees were also slightly injured. One Wealthy tree was completely girdled and looked like the Hubbardston shown in figure C referred to above.

The counts in this orchard seem also to indicate that the Hubbardston is more susceptible than the Baldwin. Forty Hubbardstons out of 100 were injured while only 15 out of 200 Baldwins were affected.

A Le Roy orchard.— A few miles from Le Roy is an apple orchard which had been set 9 years, consisting of over 400 trees of several varieties. It is a sod orchard but the trees had been growing at a good rate.

In the fall of 1906 the basal ends of the tree trunks had been incased in veneer protectors about 51 cm. high, and they were not removed until the spring of 1910. In 1908, 1909, 1910, and 1911 the grower found some of the trunks with newly diseased areas of bark, chiefly on the southwest side, from which discolored "sap" flowed in the spring and again the following July of the same season. The new cases of injury became evident in large numbers in the spring of 1910, when the protectors were removed, and again in 1911.

About 60 trees were found injured in 1910 and perhaps a similar number in 1911, for the grower said that from June 24 to 26 he had found the injury on 50 trees and by the last of July some additional ones were noticed with discolored "sap" flowing from variously discolored spots and areas of bark on the south and southwest sides.

When the orchard was visited by the writer on August 7 the general appearance of the newer, bleeding injuries as well as those which had become circumscribed by rolls of healthy callus of from one to three years' growth, was much like that of injuries studied in one of the Barnes orchards and somewhat like that in the Coldwater orchard.

Small and irregular scattered groups of cortical cells and occasionally patches of underlying phloem had evidently been injured, and in many cases the injured spots and patches had become confluent by the dying of the interspersed groups of live tissues; thus resulting in irregular dead areas in the bark which in some cases, where the underlying phloem had been injured, extended to the wood. Figure B on Plate XI shows an example on which most of the injured and dead bark has been removed with a sharp knife. In this case

some live spots were encountered on reaching the phloem and after cutting away the bark about 1 mm. deeper, irregular areas of live and dead tissues were found intermingled on the cut surface, giving it a marbled appearance. The injured spots were variously discolored from pink to blue, brown and black. A dark colored "sap" had been exuding from some very short and narrow clefts in the outer bark of this area. Some of the clefts reached even deeper than the bark had been cut away, for several of them may be seen as small ragged rifts in the cut surface.

The location of these injured patches, on the southwest side of trunks, varied considerably. They were situated anywhere from a few centimeters above the ground up to the first crotches. But the location of the maximum injury corresponded to the height to which normal bark roughening or scaling had attained on the trunk. It may also be seen in the above figure that the bark surface below and including the area of greatest injury is roughened or has begun to scale off as the bark of trees does when a certain stage in its life history is reached. Above the injured area are a few superficial checks here and there, as is commonly the case in the region of transition from rough to smooth bark. In the instance shown in figure A of Plate XI maximal injury resulted below the main crotches, about 85 cm. above ground. Here the greatest injury also occurred in the region of transition from rough to smooth bark, with a few lesser spots of exposed wood in evidence lower down. This is one of the trees found exuding discolored "sap" from injured bark covering these exposed areas in 1909. At the right and left of the stem may be seen some successful bridge grafts extending from near the ground to the branches. The tree bore a normal crop in 1911 and will probably live many years, although its lease on life has doubtless been foreshortened by leaving the exposed wood unpainted, for after the central cylinder of wood has rotted a strong summer wind will probably break it off.

It seems unlikely that the veneer around the bases of these trees had any causal relation to the bark injury, as was suggested by Stewart.[1] First, becase affected areas were mainly on one side of the trunks and were frequently above the veneer. Second, because similar new cases of injury were found in 1911 as had been observed in 1910, and affected areas seemed always to be on a region of bark that was in a certain stage of its development whether that be *under* or *above* the veneer.

It seems that the two periods of exudation of "sap" from such injured patches of bark occur during the two periods of high sap pressure, in spring and in July. Doubtless the dying, in early summer, of groups of live cells which are scattered among winter-injured

[1] F. C. Stewart Notes on New York plant diseases, I. N Y. Agrl. Expt. Sta. Bul. 328:323-24. 1910.

spots in the bark also contributes to the July period of exudation of discolored "sap." It appears as though the high pressure forces it out through the broken or dead tissues.

Another Sodus orchard.— In a 5-acre apple orchard near Sodus about 5 per ct. of the trees were found affected at the crown or crotches or both, when visited on August 9. The ground is almost level and exposed to the wind, and the soil is a clay loam which seems fairly productive. It is cultivated and cropped. The trees had been set two years and had made a good growth. They are of the varieties Wolf River and Wealthy.

The injury at the crowns had evidently been of the usual type, but the injured and partially loose bark had all died. In most cases but little regeneration occurred, although on a few trees the appearance of unevenness due to regeneration was much like that in the Clyde orchard described above. Only about half a dozen of them were completely girdled at the crown and looked like that in figure C on Plate X from an Interlaken orchard. Other affected trees were chiefly less than half girdled and had normal colored leaves.

But the most interesting phase of the injury on the trees of this orchard was confined to the crotches of the main branches. Various sized areas of dead bark were present in and around some crotches of perhaps 15 per ct. of the trees. In some instances the outer bark still appeared normal externally but when cut open the phloem region was found of a rusty brown color and disorganized into a slimy mass. In other cases the outer bark had just died and the whole affected area had a dark brown color and contained dark "sap." Many of the injured patches of bark had died and become dry and sunken below the general bark surface. On two trees Sphæropsis pycnidia had appeared on the dead bark.

On quite a number of the crotch-injured trees one or more of the most severely affected branches had died or were dying. The wood underneath the bark of such crotch-girdled branches was much stained; in fact in most cases where such a crotch-injured branch had died or had yellow foliage, the entire wood cylinder under the affected bark had become stained. The injury at the crowns was mainly on the west side, but that in and around crotches was localized with reference to the manner of branching.

In a neighboring 3- to 4-year-old sour cherry orchard one of the largest trees seemed to have the "yellows." Its foliage was said to have had a normal color a month previous, but now it looked bright yellow with here and there a tint of pink. On closer examination a slight enlargement was found on the stem about 7 cm. above the ground; although the bark below the swelling appeared normal it proved to be dead down to the ground, and the enlargement was a callus growth along the upper edge of a dead girdle of bark, very similar to the growths resulting on girdled young apple trees. The

space between the bark and the wood, along the lower edge of the callus, was filled with gum and the wood under the lower half of the girdle was stained to the depth of perhaps a centimeter. The roots of the tree appeared normal.

A Geneva peach orchard.— In a young and bearing peach orchard on the Experiment Station farm about 3 per ct. of the trees were found to have patches of injured bark on their trunks just above the ground. Three of the trees were completely girdled and more or less enlarged above the wound, like the sour cherry tree discussed above. But on most of the affected trees the dead areas of bark reached less than half way round the trunks, and the trees continued to look normal in 1911 and 1912. However, the foliage on the completely girdled ones had a yellowish tint in July and then some of it became pinkish here and there. Figure A on Plate XII is an example on which the enlargement was not very marked but where the dead region was conspicuous on account of its being smaller than the trunk above. Along the upper edge of the girdle the bark stood out from the wood and the resulting cavity was filled with gum on July 5. On other portions of the affected area the bark seemed only partially loosened by the disorganization of the phloem, and no radial clefts were present. The wood covered by the dead bark had become stained to a depth of about 5 mm.

The so-called bark-beetles (Scolytus) were boring into the bark of the completely girdled trees. On the trunk, larger branches and especially about the bases of small twigs the tiny cylindrical holes of that insect were present in large numbers, but no borer holes were seen on the normal nor on the slightly affected trees.

During early July the stumps of several young peach trees which had been similarly affected by winter-injury and borers, were received at the Experiment Station from different parts of western New York. In most of these cases a rather conspicuous enlargement or swelling occurred just above the dead girdle. No radial clefts were found in the dead bark and the gum was present under the raised portion of bark just below the callus. The phloem of the dead bark had been disorganized as in the Geneva cases and on the Sodus cherry tree, but the roots in all cases were normal as yet. The cases of injury involving less than a complete girdle always occurred on the northwest side of trunks in the Geneva orchard. There is not very much wind protection, and the trees had been growing uncommonly fast.

During late August Cytospora pycnidia, apparently the imperfect stage of *Valsa leucostoma*, were present in great numbers on the dead bark that had been left on affected trees. But no example was seen where the fungus had killed bark beyond the periphery of the original injury, as bounded by callus along the margin of areas severely affected very early in the vegetative season.

Initial injuries resulting in crown-rot produced experimentally.— Some apple trees in a seedling orchard consisting of various crosses between different varieties had been discarded, on account of undesirable qualities of their fruit. These trees were kindly given by the Horticulturist of this Station for experimental tests. The ones used for this preliminary experiment were crosses of the following varieties: Two of them were Ben Davis X Mother, germinated in 1900; one Esopus X Ben Davis, and another Ben Davis X McIntosh, which were germinated in 1899. They were set about 3 m. apart and were about the size of trees set 6 or 7 years. They bore full crops of fruit when used in the experiment on September 14, 1911.

The stem of each of the four trees used was encased in oilcloth the overlapping edge of which was sealed down with shellac. Then a sleeve from 70 to 85 cm. long was put around each trunk by rolling together a piece of galvanized iron and tying it with wire in such a way as to leave a space of about 8 cm. between it and the oilcloth surrounding the stem. Sawdust to the depth of about 5 cm. was tamped in the bottom of the galvanized iron cylinders around the bases of the trunks. About equal parts of finely crushed ice and calcium chloride were then added in alternating thin layers to the depth of 30 to 40 cm. The space above the freezing mixture was also filled with sawdust. By the time the last sawdust had been added hoarfrost had formed on the outside of the galvanized iron in the region of the freezing mixture.

Ice was piled in the form of a cone around trees 2/9 (Ben Davis X Mother) and 5/6 (Esopus X Ben Davis) up to the top of the freezing apparatus and covered with sawdust. Four long radial slits had been made in the bark of the trunks of numbers 2/9 and 5/6 before they were covered with oilcloth.

A thermometer for measuring low temperatures not being available no record of the resulting temperatures was obtained, but it seems conservative to estimate it at about — 25° C., thus allowing a loss of nearly 20° C. by conduction.

On September 16, two days after the freezing experiment was performed, about a fourth of the foliage on the treated trees was dry although still green, and much of the other drooped or curled more or less. Many of the slender bearing twigs appeared to droop more than those of neighboring untreated trees; all of which indicates that water conduction had been interfered with by the freezing of the trunks. The ice and salt in the galvanized iron cylinders had all melted, but a few lumps of ice were still present in the sawdust outside of two of them.

The effect of the low temperatures on the bark of the enclosed portion of the stems varied considerably, but it had been injurious on all of them. On tree 1/25 (Ben Davis X Mother) which measured 31.9 cm. in circumference, the bark on the northwest side had a few

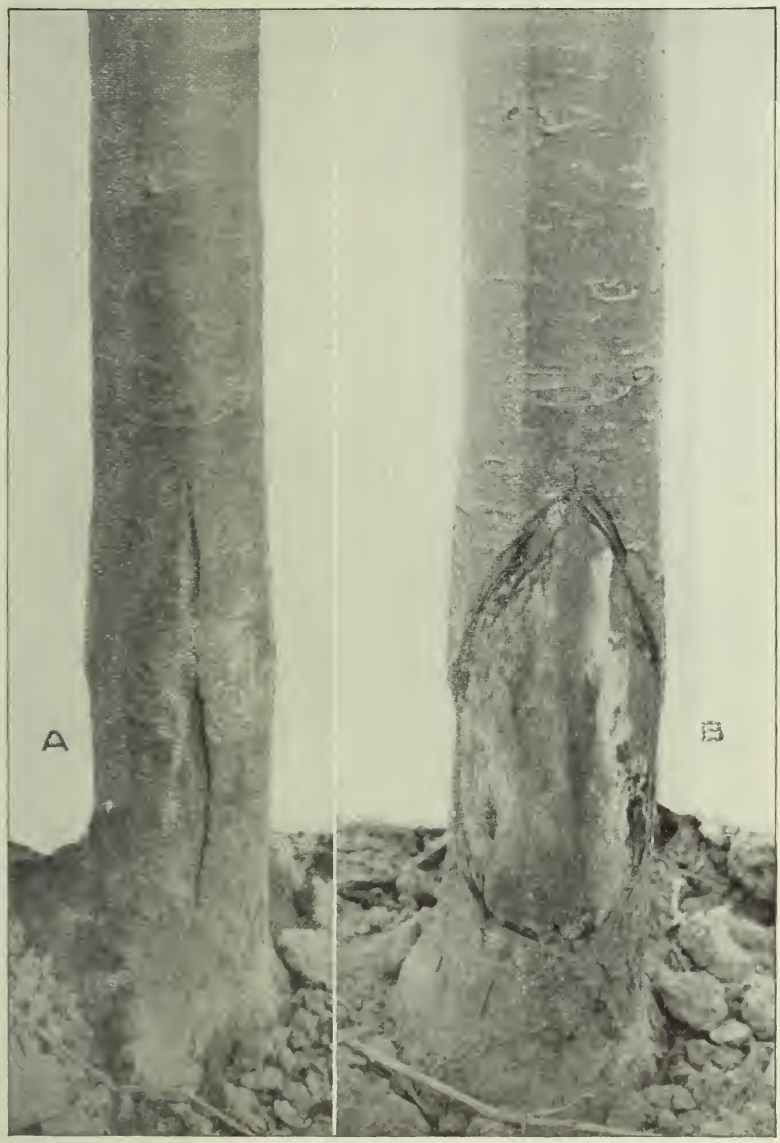

PLATE I.—STEM OF WINTER-INJURED BISMARCK APPLE TREE.

A, cleft in bark 17 cm. long on northwest side; B, nearer view of A with loosened bark removed.

(Photographed May 7 and 8, 1911.)

PLATE II.— LATER VIEWS OF TREE SHOWN IN PLATE I.

A, wax-covered wound with irregular regeneration bursting through wax; B, tree on August 15, 1911; C, grafting wax removed, showing amount and distribution of regeneration, September 11, 1911.

PLATE III.— WINTER-INJURED TREES FROM A CLYDE ORCHARD.

A and B, tree before and after removal of loose and dead bark, showing warty nature of regeneration; C and D, extension of injury by subsequent death of bark above externally evident injury; E, practically all injured bark on this tree was loose and dead when photographed June 3, 1911.

PLATE IV.— WINTER-INJURED TREES FROM CLYDE ORCHARD.

A and C, injured bark removed and wood covered with grafting wax; B, stump of girdled tree with sprout grown from it.

(Photographed August 3, 1911.)

PLATE V.— WINTER-INJURED APPLE-TREE STEMS FROM CLYDE ORCHARDS.

A, trunk that had been surrounded by veneer protector; B, large depressed area on northwest side, still green-colored on June 18, 1912; C, tree injured in winter of 1910–11, as it appeared on June 18, 1912.

(A, photographed August 3, 1911.)

PLATE VI.— WINTER-INJURED TREES FROM WEEDSPORT ORCHARD.

A, sprout grown from Baldwin stump; B and C, stems with exposed wood painted.

(Photographed August 4, 1911.)

PLATE VII.— VIEWS IN WEEDSPORT ORCHARD.

A, row of Baldwin in center, Ben Davis on both sides; B, entire row of Baldwin stumps in center replaced by young trees.

(Photographed, A on August 4, 1911, and B on July 23, 1912.)

PLATE VIII — WINTER-INJURED TREES FROM GLENS FALLS ORCHARD.

A, tree with short cleft, but girdle of loosened bark extending about half way up to A. B, another tree with loosened bark removed; had been cleft about half its length.

(Photographed June 24, 1911.)

PLATE IX.— WINTER-INJURED TREES FROM ANOTHER GLENS FALLS ORCHARD.

A, tree with about two-thirds of bark loosened from trunk; B, tree with bark cleft about 2.5 dm. long, bark loosened up to lower crotches.

(Photographed June 24, 1911.)

PLATE X.— WINTER-INJURED TREES FROM INTERLAKEN ORCHARDS.

A, tree with callus-bordered area of dead wood on south side; B, inadequately cleaned and painted wound; C, tree completely girdled, dead bark partially adhering, thick callus along upper edge of girdle.

(Photographed August 5, 1911.)

PLATE XI.— WINTER-INJURED TREES FROM LE ROY ORCHARD.

A, bridge grafts at right and left of girdled stem; B, injury resulting from winter of 1910–11, apparently an early stage of the type shown in A.

(Photographed August 7, 1911.)

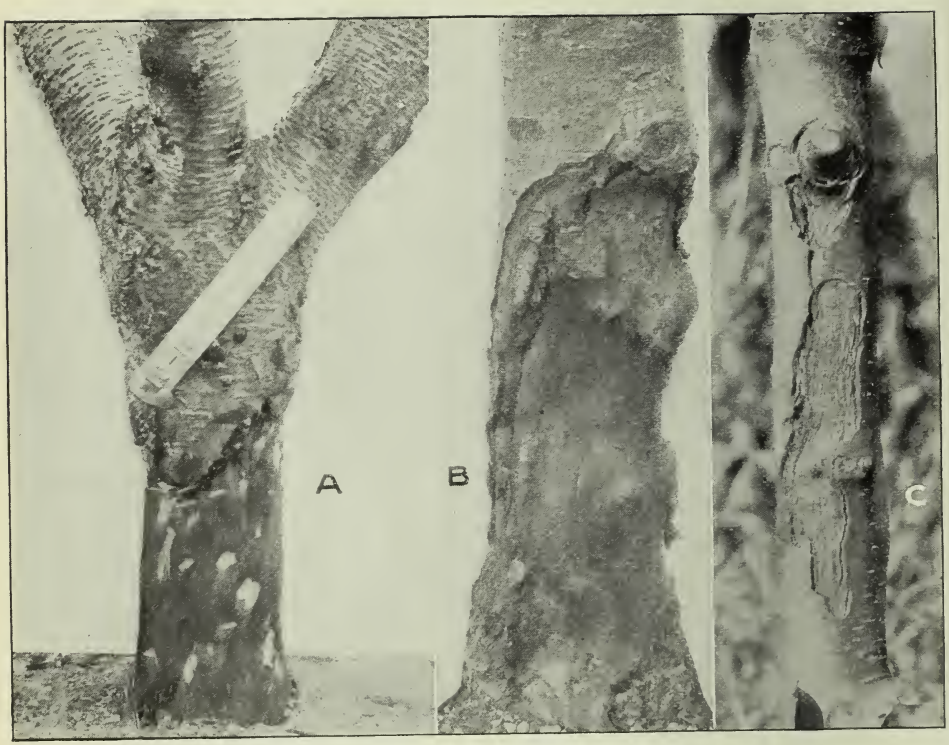

PLATE XII.— WINTER-INJURED FRUIT TREES.

A, peach tree with dead girdle of bark removed, showing callus along upper margin; B, typically crown-rotted apple tree, with girdling nearly complete; C, Bartlett pear tree with depressed dead areas where inner bark was severely winter-injured.

(Photographed, AT GENEVA, A on July 5, 1911; B, in August, 1911; at Medina, N. Y., C on August 16, 1911.)

PLATE XIII.— CROWN-ROT SCARS ON OLD APPLE TREES.

A, old wound surrounded by irregular regeneration; with a second injury dating one year after first one showing below at left; B, advanced stage of "heart-rot" following winter-injury.

(Photographed at Geneva, 1911.)

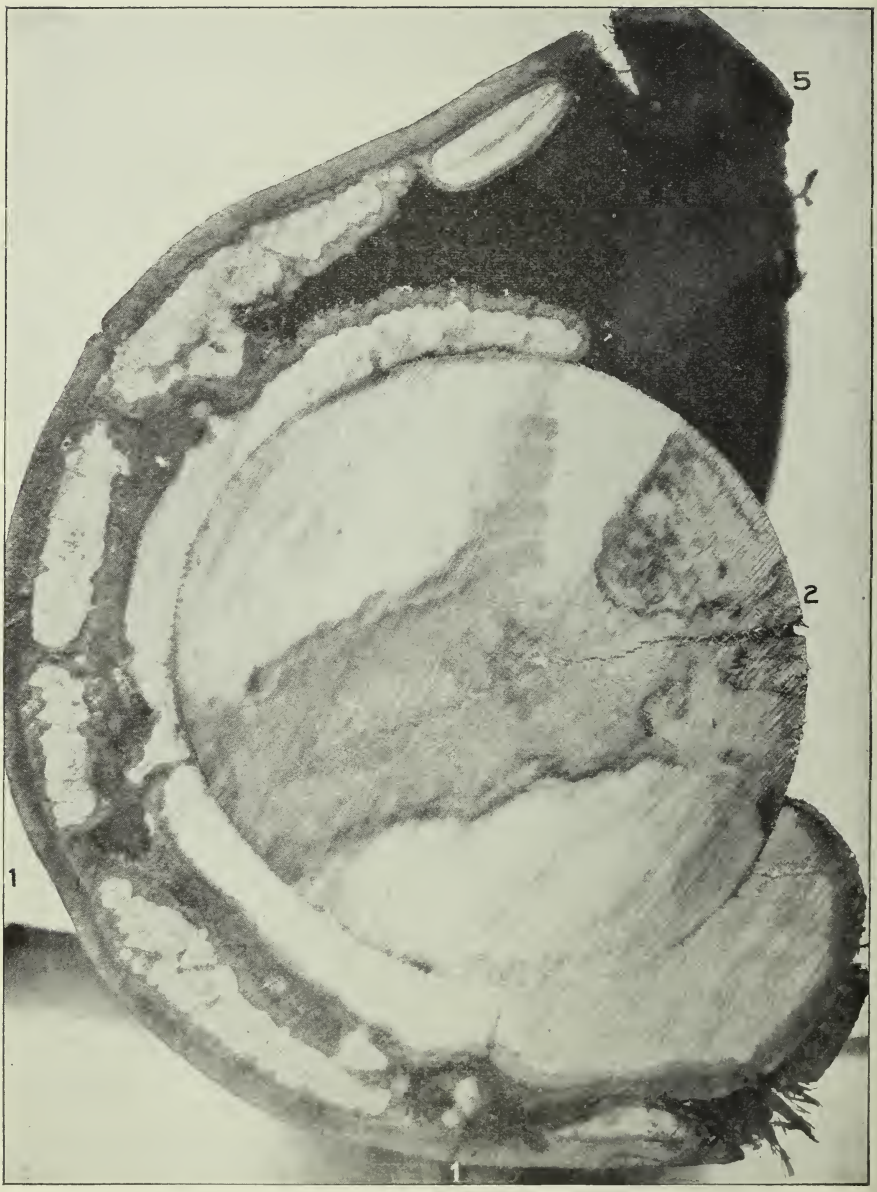

PLATE XIV.— *Acer platanoides* INJURED IN WINTER OF 1909–10.

Section of trunk about 25 cm. above ground. Initial injury forms circle with exposed wood. Wood partially decayed by a Polyporus.

(Cut and photographed November 25, 1910.)

PLATE XV — *Acer platanoides* INJURED IN WINTER OF 1909-10.

Section taken 10 cm. above that on Plate XIV. Specimen kept in museum from November, 1910, to July, 1912; "heart-rot" progressed; numerous Cytospora pycnidia found on old dead bark at left, between 3 and 6.

(Cut and photographed July 3, 1912.)

PLATE XVI.— WINTER-INJURED MAPLE TREES.

A piece of trunk shown in section on Plates XIV and XV; 4, point at which section in Plate XV was cut. B, *Acer platanoides* with closed injury on west side.

(Photographed, A on November 25, 1910; B in 1911.)

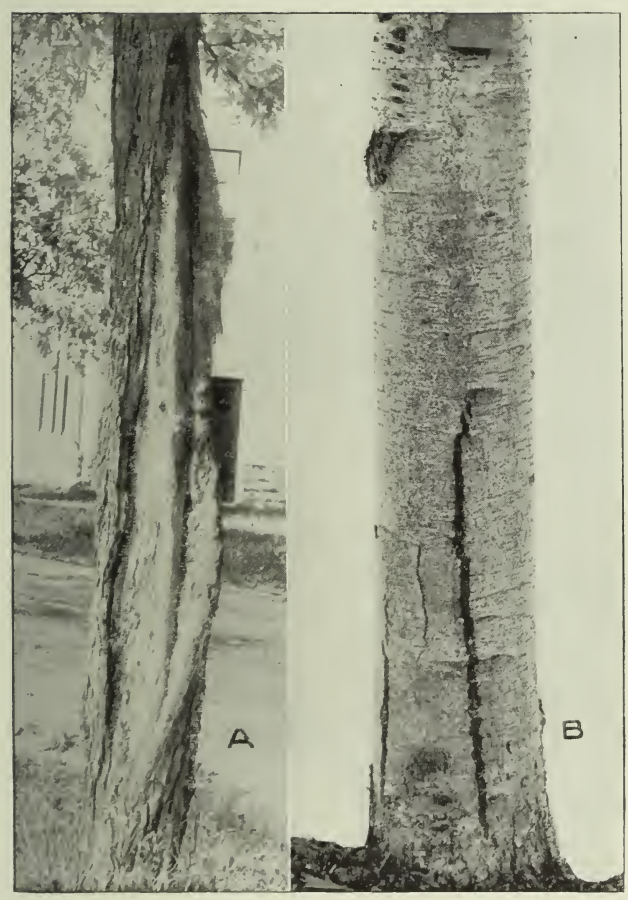

PLATE XVII.— WINTER-INJURED TREE TRUNKS.

A, *Acer saccharinum* with injury on southwest side, occurring in winter of 1909–10, followed by double regeneration like that shown in Plates XIV, XI B, and XVI; B, apple tree trunk with cleft in bark, from winter of 1910–11.

(Photographed, A on July 2, 1911, and B on July 23, 1911.)

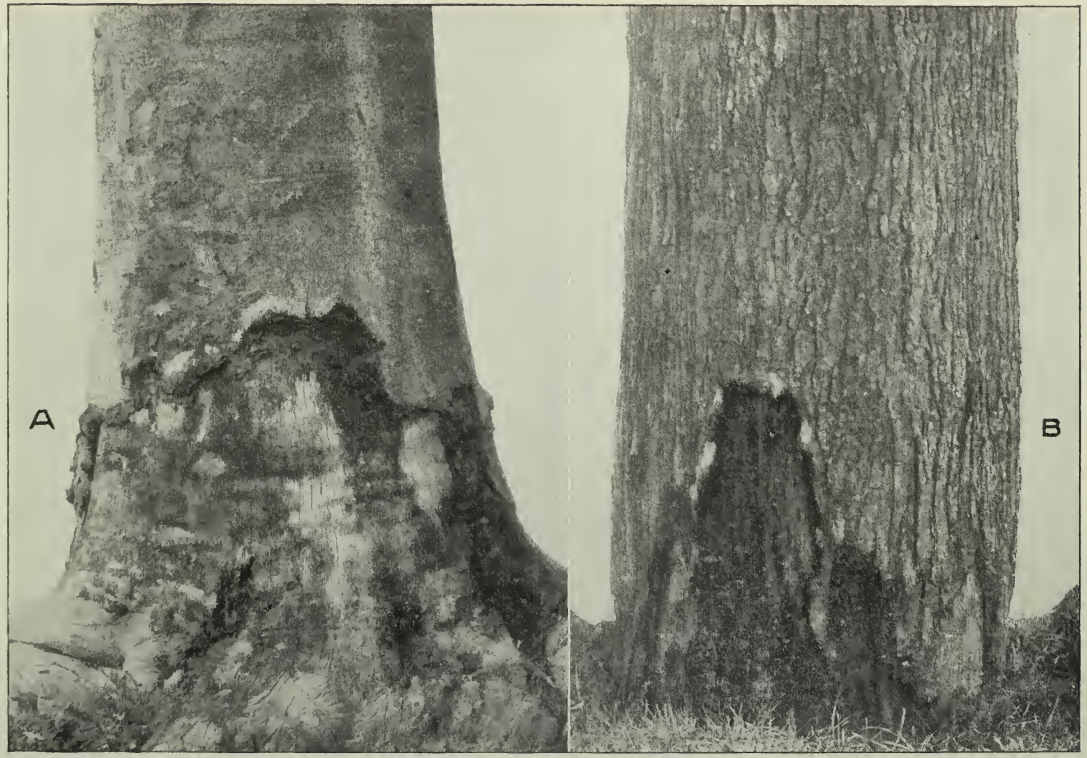

PLATE XVIII.— CROWN-ROTTED SHADE TREES.

A, weeping beech on which initial injury occurred in winter of 1909–10; tree was girdled and died (1911); B, *Acer saccharinum*, 45 years old, affected on northwest side; stands on east and west street.

(Photographed in July, 1911.)

PLATE XIX.— CROWN-ROTTED MAPLE TREE TRUNKS.

A, *Acer platanoides* with injury on west side; stands on an east and west street. B, *Acer platanoides*, showing injury to have taken place in three stages or seasons, on west side of tree on southeast street corner. C, *Acer saccharinum*, affected on south and north sides; failed to leaf out in 1912; stands on northeast street corner.

(All photographed in July, 1911.)

PLATE XX.— A COMMON TYPE OF CROTCH CANKER.

Acer platanoides on an east and west street, with canker, A, on east side about 1.7 m. above ground; circumference at twig below canker, 81.3 cm.

(Photographed in July, 1911.)

PLATE XXI.— WINTER-INJURED MAPLE TREE TRUNKS.

A, *Acer saccharinum* in a wind-swept lawn, midway between two large buildings which stand to east and west; injuries occurred on both north and south sides; tree failed to leaf out in 1912. B, *Acer platanoides*, on north and south street, with injury on north side showing double regeneration and exposed wood below; another injury near base (at X) occurred in winter of 1910–11.

(Photographed, A in July, 1911, and B in July, 1912.)

PLATE XXII.— SECTIONS OF WINTER-INJURED *Acer Negundo* FROM MADISON, WIS.

1, cut about 15 cm. above ground, loosened bark at left; 2, cut 2.5 cm. above 1, slight cleft in bark and trace of one in wood; 3, cut 2 cm. above 2, clefts more marked; 4, cut 2.5 cm. above 3, loose bark nearly all dead and callus conspicuous.

(Tree cut May 28, 1912; photographs made in Geneva July 25, 1912.)

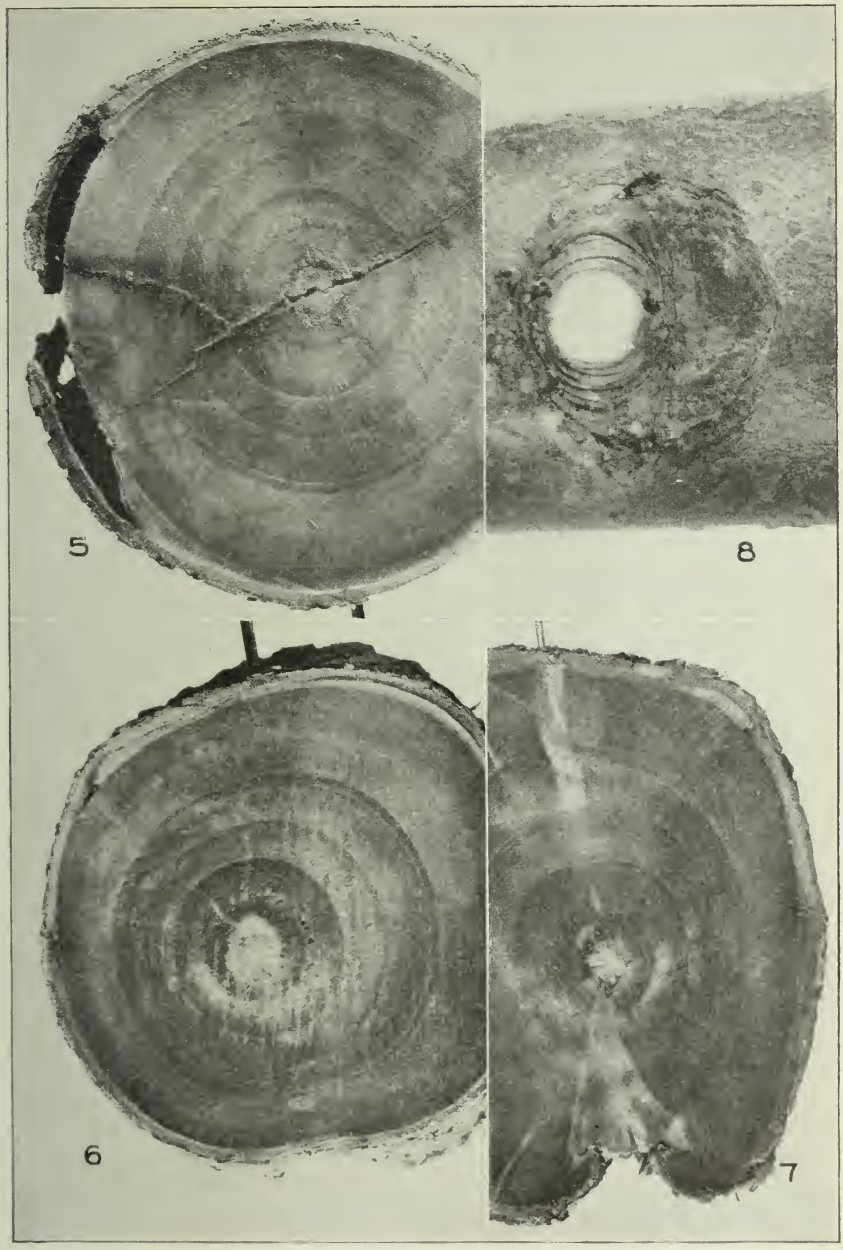

PLATE XXIII.— SECTIONS OF *Acer Negundo*; PIT CANKER ON APPLE BRANCH.

5, cut about 45 cm. above 4, Plate XXII, cleft extending through wood; 6, cut about 15 cm. above 5, which is about 1 cm. above upper end of cleft in bark; 7, cut 3.2 cm. above 6, about 5 cm. below first crotches; 8, "pit canker" around hole made by bark beetle (Scolytus) at base of twig on tree frozen artificially in September, 1911.

(Photographed in July, 1912.)

scattered brown blotches from about 3 cm. above ground up nearly 12 cm., but no clefts had resulted. On removal much of the phloem in that region, about half way round the stem, had a brown color, although there were groups of normal colored phloem tissues scattered along in the affected area. Most of the cortex, aside from the blotches referred to above, was of normal color and apparently uninjured. The whole discolored area of bark was removed and the wood underneath it was found to have a normal color also.

Tree 2/9 (Ben Davis X Mother) having a circumference of 36 cm. and on which the bark had been slit, sustained injuries of a different type, but they were also confined to the lower portion of the trunk. The evident injuries were on the north side and did not extend more than 16 cm. above ground, but there were no clefts aside from the slits made before. A strip of bark on the north side between two of the slits which were about a fourth of the circumference of the trunk apart was loosened from 6 cm. above ground up 7 or 8 cm. It was of normal color except on its inner side where it was slightly browned. The wood surface underneath had a similar appearance. The bark on the west side was also removed but no discolored spots were found in it; however, the edges of the bark along the slits were loose.

On tree 3/12 (Ben Davis X McIntosh) which had a circumference of 34.3 cm. the bark appeared discolored on the southwest side over an area of about a third of the circumference and from 5 cm. above ground up 7 or 8 cm. In this case there were only a few small groups of cortical cells in the whole discolored area that were of normal color. Practically the entire phloem was also discolored but the wood underneath appeared normal. The tree was cut off and no discoloration was evident in the wood. However, a few spots were found in the phloem and inner cortex. The injury was confined to the basal 15 cm. of the trunk.

Tree 5/6 (Esopus X Ben Davis) measured 30.5 cm. in circumference. The bark was loosened very slightly on the west side along one slit and it had a discolored area on the northwest side covering about a third of the circumference. This discolored patch was similar to that described on tree 1/25, except that there were fewer and smaller normal colored spots in its interior. The discolored and loose bark was removed and the wood underneath appeared normal. The injury was all within 15 cm. of the ground.

September 18, four days after treatment, hundreds of the bark-beetles (Scolytus) were present on the remaining three trees, although none could be seen on the surrounding untreated ones. They were especially numerous on trees 1/25 and 2/9 where large numbers of small round holes had been dug or were being dug into the bark of stems, main branches, and around the bases of small twigs and spurs. It seemed as though each hole was bored by a pair of the beetles; at any rate, in instances where a beetle was actually boring

there was always one waiting at the rim of the hole. Tree 5/6 was infested with fewer beetles, and since the injured trees were about the same distances apart and similarly surrounded by normal ones, it appeared to indicate a difference in the trees which had been treated. As a matter of fact there was a slight difference in the amount of dead foliage on the treated trees: tree 5/6 had least and tree 2/9 had most of it. But nearly a third of the foliage of the latter tree was still living and appeared more turgid than it did two days before.

Since the bark on the treated trees was all alive and of normal color (except some on the trunks near the ground) it is difficult to understand how these beetles selected only the injured trees on which to bore, unless it be by differences in turgidity or water content of the bark though the presence of clumps of dead leaves may have been a sign for attack.

In this connection it may be of interest to note that D. H. Jones has recently published an article[1] on *Scolytus rugulosus* as an agent in the spread of bacterial blight of pear trees.

OBSERVATIONS DURING 1912.

The field observations during 1912 were mainly of a supplementary nature. Observations had shown that the types of winter-injury under consideration, which may result in crown-rot and cankers, occurred sometime between the middle of November, 1910, and the first part of January, 1911; also that after thawing and during the following vegetative activities injured tissues become discolored and more or less disorganized, leading to discoloration and death of some adjoining and of all isolated live tissues. During the winter of 1911–12 and the following summer, an effort was made to localize more definitely the time of occurrence of these injuries in winter; to study the histological modifications induced, and to make further observations on trees injured in winter of 1910–11.

During last scholastic year observations were made in and around Madison, Wisconsin, and after the first of June were continued in New York. The histological study begun in the University of Wisconsin had to be discontinued after arriving at the Experiment Station on account of lack of necessary apparatus; but the field observations and the fixing of material for the histological study were continued. Since the material yet remaining necessary for a histological study of the initial injuries and the subsequent changes taking place in affected areas had been fixed and infiltrated with celloidin, the gaps in the studies made in the University of Wisconsin

[1] D. H. Jones. Scolytus rugulosus as an agent in the spread of bacterial blight on pear trees.
Phytopath. 1:155–58. 1911.

and the further extension of the histological series into the early summer may be finished at odd times so that its publication should not be long delayed.

Late fall and early winter were rather rough and cold around Madison. The average temperature for both October and November was below the normal. But December was an uncommonly mild month with the temperature ranging considerably above the normal, except during the last few days when it sank to — 22.2° C. January was colder than any previous January on the records of the United States Weather Bureau for that region; the temperature was over 8° C. below the normal. The highest temperature was — 0.55° C. and the lowest — 31.6° C. The temperature in February was also 2.7° C. below the normal. The highest temperature during the month was — 7.7° C. and the lowest — 27.3° C. The March record was 3.8° C. below normal, with a minimum of less than — 20° C. Many periods of high winds occurred; one of the strongest lasted most of the day on December 10 with a maximum during the middle of the afternoon of 56 miles per hour, and exerting a force of nearly 17 pounds per square foot.

In western New York the weather was very similar to that described for the Madison region except that it was not quite so cold.

At Madison three small apple orchards on the university farm were easily accessible for observation and study. One had been set about two and another about six years; while the third orchard consisted chiefly of trees that had been set some 9 to 14 years. In the oldest orchard many trees of undesirable varieties were kindly put at the writer's disposal by J. G. Moore, Professor of Horticulture in the University. The trees in these orchards were examined several times during the winter in order to determine if possible the time of injury.

Only three cases of crown-rot were found, and those were on trees in the oldest orchard. The initial bark injury seemed to have occurred during the winter of 1909–10, as judged by the presence of callus of two years' growth around the wounds. In no instance, however, did the injury extend more than half way around the base of the trunk.

Effect of changes in temperature on the circumference of tree trunks.— In order to secure some first-hand data on the effect of low temperature on the circumference of tree trunks a small number of apple trees in the oldest orchards were measured at different times. The regions around which the measurements were taken were marked at two points on each tree in order that the different measurements would be more comparable. They were made with a steel tape-measure which when used during low temperature naturally somewhat minimized the results of the contraction of the tree trunks.

The following table shows the changes in circumference induced by changes in temperature:

TABLE I.—CHANGES IN CIRCUMFERENCE OF APPLE TREE TRUNKS DURING WINTER.

DESIGNATION OF TREES	Circumference Nov. 8, 1911 temperature 6.6° C.	Circumference Nov. 13, 1911 temperature — 9.4° C.	Circumference Jan. 6, 1912 temperature — 28.3° C.	Circumference April 6, 1912 temperature 21° C.	Maximum shrinkage of circumference
	Cm.	*Cm.*	*Cm.*	*Cm.*	*Per ct.*
3/15	71.3	71.0	69.6	71.0	2.3
5/23	64.9	64.6	63.5	64.6	2.1
7/28	43.8	43.5	42.4	43.6	3.1
7/21	48.6	48.3	47.6	48.5	2.0
9/25	59.5	59.1	58.4	59.5	1.8
9/23	51.1	50.8	50.1	51.0	1.9
9/22	51.8	51.5	50.8	51.7	1.9
11/21	67.6	67.1	66.4	67.6	1.7

As may be seen in the last vertical column of this table the percentages of decrease in the circumferences of the 8 tree trunks varies from 1.8 to 3.1, and the average is a little over 2 per ct. It is also interesting to note that the contraction resulting from a change of temperature from 6.6° C. to — 9.4° C. is not as great per degree of change in temperature as it is in the range from — 9.4° C. to — 28.3° C. In the first case a change of 16° C. resulted in an average contraction of only a little over 3 mm. or 0.19 mm. per degree of change; while in the second an additional change of 18.9° C. caused a contraction averaging 8.8 mm. or 0.46 mm. per degree of change. The measurements taken on April 6, although made at a higher temperature than those of November 8, averaged less; and none of them were above the first November measurements. Possibly that is an error of observation, yet it may indicate a slight diminution in size due to a loss of water during winter.

Tree trunks cleft open during the first excessive cold period.— The excessive cold period in the winter of 1911–12 came during the first week of January with a minimum temperature of — 31.1° C. On January 6 quite a number of tree trunks in the above apple orchard were cleft open a little above the ground and others in the lowest crotches. The clefts in the trunks were from 2 to 5 dm. long and from 5 to 15 mm. wide. They extended at least to the pith as shown by the insertion of a sharpened stick. The clefts were mostly on the north and west sides. The crotch clefts were always at right angles to the branching and usually widest above, appearing as though the crotches had been split by driving in a thin wedge from

above. In two instances where measurements were taken the component parts of the crotches had separated about 2 cm., which seems to indicate that there had also been a longitudinal contraction of the outer portions of the trunks, thus resulting in an outward bending of the branches. The bark, however, had not separated or loosened from the wood along the clefts.

Many shade trees in the city of Madison, including maple, oak, catalpa, linden, etc., also had their trunks cleft open during the first week of January. Frequently a long cleft extended from near the ground up as high as 2 meters, and one or more shorter ones from near the ground up about 5 dm. or less. The longer clefts went at least as far in as the center of the trunk. No bark had been loosened.

Changes in circumference of cleft trunks due to rise of temperature.—Some measurements were taken on linden and catalpa trees on January 6 when the thermometer stood at — 28.3° C., which are recorded in the table below. In this case, also, errors that may have arisen due to the contraction of the steel tape-measure would result in giving higher values to the measurements at low temperatures and thus give slightly lower percentages of increase in the circumference when compared with those taken at higher temperatures. The measurements were made at marked points on the trunks and also included the width of the clefts. When the second measurement was made the clefts had all closed tightly. The change in circumference that

Table II.—Measurements on Shade Trees in Madison which Were Cleft During the Winter of 1911–12

Designation of Trees	Circumference Jan. 6, 1912, temperature — 28.3° C.	Location, width and length of clefts.			Circumference April 6, 1912, temperature 21° C.	Increase in circumference	
	Cm.		*Cm.*	*M.*	*Cm.*	*Per ct.*	*Per ct.*
1. Linden...	197.5	South side..	2.0	2.4	202.0	2.2	3.3
2. Linden...	186.7	West side..	1.6	2.5	189.6	1.5	2.4
3. Linden...	199.2	Southwest..	2.0	2.4	202.8	1.8	2.8
4. Linden...	159.0	Southwest..	2.2	4.0	161.9	1.8	3.2
5. Catalpa..	67.3	Southeast...	1.8	0.7	68.6	1.9	3.2
6. Catalpa..	42.8	Northwest..	1.3	0.7	43.8	2.3	5.5
7. Catalpa..	40.0	West side...	1.3	1.0	40.9	2.2	5.6

these tree trunks underwent between January 6 and April 6 was therefore greater than is shown by the measurements on which the first column of percentages is based. After deducing the width of the clefts from the measurements made on January 6, the percentages of increase in the circumferences are appreciably higher, as shown in the last vertical column.

An experiment in rapid thawing and swaying of apple trees. — On January 8 about 5 liters of water with a temperature of nearly 60° C. was poured in splashes on the lowest crotches of each of two apple trees in the oldest orchard, and allowed to run down the trunks. Immediately after application of water the trees were swayed vigorously during about a minute. It was in the afternoon and the temperature had risen to about — 26° C. The trees were of normal appearance. One of them had been set about 9 and the other about 12 years. Perhaps a liter of water was left after the second tree had been treated and was let stand in a tin vessel in the snow while the tree was being swayed. The remaining water was then splashed on to the lowest crotch of another large apple tree and ran down its trunk. On another tree two branches about 2.5 cm. in diameter were bent downward, considerably, a number of times, but not far enough to cause any audible breaking.

By testing with a knife it was found that the bark and a little of the outer wood had been thawed by the hot water, but a few minutes after the application of water had been made the whole wet surface was coated with a thin sheet of ice. One of the swayed trees was sawed off about 20 cm. above ground and carefully examined for indications of discoloration or injury, but none could be found in any part of its trunk.

About the middle of March, after all aerial portions of trees had been thawed for several days, practically the entire bark of the remaining tree-trunk receiving the hot water treatment in January had died and become brown. There were only a few small blotches of green colored outer cortex here and there that seemed to be alive. The whole phloem as well as the outer surface of the wood had become discolored over all parts of the tree where the hot water had been applied. On the inner side of the branches as much as 2 dm. above the crotches the bark had all died and become brown. All the bark on the stump of the tree that had been sawed off was also dead and brown to the ground. On the side on which most water had been splashed the bark was dead below the surface of the ground and around the bases of some roots.

The bark had not become loosened on the trees given the hot water treatment but considerable disorganization had occurred in the phloem region. Above ground the injured bark seemed to have dried out a little, but underground and at its surface the affected bark was full of brown " sap." About a fourth of the bark of the tree on which the last water had been poured was also found dead. The greatest effect occurred in the crotch and over an irregular area extending down the trunk about 3 to 4 dm. on the side receiving the hot water.

The bark on both of the branches which had been bent downward was partially loose on the upper side, and dead over a length of about

2 dm. where the bending had occurred. The general appearance was that of so-called "sun-scald." The affected bark was rusty brown in color and the phloem region was much disorganized.

Effect of low temperature on the diameter of apples and potatoes.—On January 8 some medium-sized potato tubers and apples were carefully measured with a caliper by adjusting it against the heads of two pins which had been stuck into the specimens at opposite points of the greatest diameter, up to the heads. Afterwards they were placed out of doors and left there over night with the temperature ranging around —27° C. Early the next morning one was taken in at a time and remeasured over the pin-heads. The data are recorded in the following table:

TABLE III.—CHANGES IN DIAMETER OF APPLE FRUIT AND POTATO TUBERS INDUCED BY LOW TEMPERATURE

Apple No. 1,	diameter	in	evening	77.5 mm.,	in	morning	77.4 mm.
Apple No. 2,	"	"	"	77.3 mm.,	"	"	77.0 mm.
Apple No. 3,	"	"	"	78.0 mm.,	"	"	77.9 mm.
Tuber No. 1,	"	"	"	96.5 mm.,	"	"	97.0 mm.
Tuber No. 2,	"	"	"	98.7 mm.,	"	"	100.2 mm.
Tuber No. 3,	"	"	"	88.0 mm.,	"	"	89.5 mm.
Tuber No. 4,	"	"	"	114.0 mm.,	"	"	116.5 mm.

This shows an average decrease in the greatest diameter of the apples of 0.16 mm. or about 0.2 per ct.; and an average increase in the longest diameter of the potato tubers of 1.75 mm. or about 1.7 per ct.

Discoloration, after thawing, of structures injured by low temperatures.—Winter- or low-temperature injured plant structures can usually not be recognized as being injuriously affected until after they have thawed and become discolored. However, in some instances injuries may be seen by microscopic examination immediately after thawing or even while frozen. Some of the potatoes and apples used in the above experiment were placed outside an east window and the others were thawed and cut in pieces for microscopic examination and for observing the development of discoloration in air, water, etc.

The thawed tubers were very soft but of normal color. The "sap" could be squeezed out with the hand as readily as water from a sponge. By using thin hand sections it was easily seen that many of the cells had been broken and others separated along the middle lamellæ. The thawed apples were soft also but not spongy like the tubers. Fewer cells were broken and separated, in fact at the calyx end there was practically no indication of injury.

Pieces of tubers were placed into 15, 30, 50, 70, 80, 95 per ct., and absolute alcohol; in distilled water, and into 2 per ct. formalin. Pieces of both apple and potato were exposed to the air over night.

On the following day the pieces of potato exposed to air had become

very dark brown on the outside, and the pieces of apple had a rusty brown color throughout except around the calyx end, where there was still some normal colored tissue. The pieces of potato in water were almost as much discolored as those in air; and those in the lower alcohols seemed to have a very faint muddy-yellow discoloration. About a week later the pieces in the alcohols from 15 to 70 per ct. had all changed to a light brown or black color, while those in 80 per ct. were but slightly discolored. The pieces in 95 per ct. and absolute alcohol, and in 2 per ct. formalin had remained unchanged.

Crotch and other bark injuries observed in Madison orchards.— In the three University orchards described above much bark injury occurred during the winter of 1911–12, and presumably during the excessive cold weather in January, because no injury could be found in the latter part of November or before the steady cold weather began. The fact that both bark and wood clefts occurred in the first week in January makes it appear plausible that the bark injuries in crotches and other parts of trees occurred at least not later than that date; and, as will appear in another connection, it seems likely that this injury also occurred at this time. At any rate, when the orchards were examined again late in March and early April many trees were found having very severe injuries in the inner bark of crotches and callus growths around old wounds.

About a fourth of the trees in the apple orchard which had been set about two years had the inner bark of several crotches more or less severely injured, although no indication of injury could be seen outside. The cortical tissues were nearly all green and normal looking while much of the phloem was discolored and often had a region near the cambial zone where a disorganization or partial separation had occurred in such a way as to partially or even completely loosen the bark in various sized areas or patches.

No difference in general appearance could be detected between injured and sound trees, nor could the injured crotches be told from those not affected. The injury was chiefly confined to the main crotches and the bases of one- and two-year-old, ascending shoots coming from the lower parts of larger branches. The affected areas in the larger crotches involved the angle of the crotch and the inner side of both component branches up to various heights, depending upon the size of the affected region.

In the most severe cases the affected area usually involved from 2 to 3 cm. of bark on the inside of the branches of a crotch as well as that surrounding its angle to as much as 4 cm. below.

The injury around the bases of ascending shoots originating from dormant or adventitious buds on the larger branches was much like that in the crotches and was always most severe and sometimes even confined to the bark in the lesser angle between the shoot and branch from which it grew. In many instances, however, the inner bark or

phloem region was affected all around the base of shoots, and to such a degree as to be almost entirely loosened in a girdle one or more centimeters in width. The affected region in the phloem had a rusty brown color just like that in injured areas of the larger crotches. But there were no radial clefts in any of the affected regions.

Probably more than half of the trees in the other young apple orchard which had been set about 6 or 7 years were crotch-injured. In this case, however, the affected areas in the larger crotches often involved the bark on the inner side of the branches and below the angle to the extent of 8 to 10 cm. The injuries around the bases of young ascending shoots on these trees were also more extensive and severe than in the above younger orchard.

There were probably a half dozen of the large trees in the oldest apple orchard (set 8 to 14 years) found injured in the crotches, but only on two of them had the injury been severe. In these cases areas of bark as large as a man's hand were sufficiently affected in the phloem region to be partially loosened. In another instance the bark on a normal looking callus surrounding an old crown-rot scar was also much discolored in the inner phloem and looked as though it had been sufficiently isolated by the affected tissues to result in the death of the entire outer bark over the injured area, thus probably giving rise to a canker-like region of successive stages of development as is shown on maples in figures B of Plates XVIII and XIX, and to a less degree in figure A of Plate XIII. A similar injury was also found on callus growths of large sour cherry trees on the north side of this apple orchard.

In none of the cases of this type of bark injury in any of the orchards could a trace of radial clefts be found. The loosening of the bark seemed to have been brought about by injury or partial rupture in the inner phloem thus isolating the bark from the wood.

Since it had proved difficult to prepare sections for microscopic study from such injured regions of large trunks and branches on account of the fact that the injured bark usually drops off before pieces small enough for fixing and sectioning can be cut, suitable sized pieces were prepared from the basal parts of some of the injured one- and two-year-old shoots in the two younger apple orchards. Pieces of typical examples of such shoots were fixed and infiltrated for sectioning at intervals until the latter part of May, and will be made the chief basis for a subsequent report on the histological modifications resulting in crown-rot and certain types of cankers.

By the first part of May it became evident that most of the larger areas of injured bark in the crotches of the apple trees were dying. Various sized brown spots appeared on the outer surface of the most severely affected regions and it was found that these places of external browning were only an extension of the internal discoloration of disorganizing phloem. By the latter part of May many injured

areas both in the main crotches and around the basal ends of ascending shoots on large branches had died completely, and around the periphery of the region a thin callus ridge had formed under the bark. In cases where the affected areas extended some distance up the branches above the crotch the general appearance was surprisingly like that of ordinary cankers, because the dead area had become sunken and usually a fissure had begun to form around them like that shown in figure C on Plate XII and figure B of Plate V.

The wood underneath the injured bark had become very much stained. Even in cases where the injury was not severe enough to cause the death of the outer bark, the wood was stained to a considerable depth. Many of the shoots a centimeter or more in diameter had the entire wood cylinder stained a rusty or dark brown in the affected region in case the bark injury had been severe all around their bases. In instances where the bark was injured chiefly on one side, only that half of the wood cylinder had become stained. It appeared as though there had been a diffusion of a stain or of an active discolorizing agent from the disorganizing phloem into the adjoining wood, very much like that observed many times in a number of orchards during early summer of 1911. The crotch injuries found in a Sodus orchard discussed on page 30 were apparently later stages of this type of injury.

Radial clefts and loosening of bark occurring together.— On a number of thrifty young maple trees from about 5 to 15 years old, along some streets in the western part of Madison, the bark alone and on others both bark and wood were cleft open during the first week of January. The clefts were mostly on the west side, even though some of the trees were along streets going north and south, but since that portion of the city is not closely covered by houses the speed of the west wind is checked but little. The bark on a few of the trees on streets going north and south was cleft either on the north or south side. None of the bark appeared to be loose when examined in January shortly after the occurrence of the clefts, but possibly that was due to the fact that the trees were frozen solid. They were re-examined in early spring and found to have loose bark on both sides, as well as some distance above and below the ends of the clefts.

The clefts varied in length from about 1 to 6.5 dm. and were most common on *Acer platanoides* and *Acer Negundo*. In cases where the clefts in the bark were short the wood underneath was not cleft but in those which were 3 dm. or more in length the wood was usually also cleft more or less. On some vacant land near the western edge of the city was a small clump of *Acer Negundo*, on the west side of which was one having the bark and wood both cleft. The cleft in the bark was 6.4 dm. long and was entirely closed in early spring while the sap was flowing, but by the latter part of May it stood open about a centimeter in the middle. The tree was sawed off on May

28 and short sections were taken from the typical regions and preserved in alcohol until they could be photographed. Figures 1 to 7 on Plates XXII and XXIII show sections of some of these injured regions.

Figure 1 is a section about 15 cm. above ground and 2 cm. below the lower end of the cleft in the bark. Its greatest diameter is 7.6 cm. In a section taken about 3 cm. nearer the ground there was but a trace of loosening of the bark, while in one taken 2.5 cm. above 1 the loosened area is wider and there has been an appreciable growth of callus along one edge. In the section shown in figure 1 the bark was not entirely loose; the injured region in the inner phloem seemed to offer slight resistance to the removal of the bark. The isolated bark was almost of normal color in figures 1 and 2 except at the margins of the cleft of 2. In figure 3 which was taken 2 cm. above 2, the loosened bark was slightly discolored and seemed to be nearly dead. The cleft in the wood shown in figure 3 to begin near the cleft in the bark and extending toward the pith through two annual growths may also be seen in figure 2. In figure 4 the bark above had died while that below the cleft was still partially alive. Figure 5 is taken above the middle of the cleft and shows the typical appearance of the region of maximum injury of both bark and wood. The loose bark is entirely dead and stands out away from the wood owing to the callus growths around the periphery of the wound and to the entire lack of adherence between the bark and wood. At the lower left of the figure some regeneration had occurred on the surface of the exposed wood.

In the region of the trunk about midway between the ends of the cleft in the bark the wood had been split through the pith to the inner side of the bark on the opposite side, but leaving the bark uninjured. A rather conspicuous ridge of new wood had developed over the end of the wood cleft; it was about as thick as the callus growth at the lower left margin of the loose bark shown in figure 5.

Figure 6 is taken about a centimeter above the upper end of the bark cleft and 15 cm. above figure 5. Here as in figure 1 no cleft resulted in the wood and the partially loosened bark was still alive. Figure 7 is of a section 3.2 cm. above that shown in figure 6 and about 5 cm. below the main crotches. The strip of injured bark is slightly wider at this point but is of normal color and seems to have grown in *thickness* by the development of new wood on the inner side of the bark against last year's growth.

As these figures plainly show, the increase in the diameter of this tree trunk in 1911 was nearly as much as that of any two previous seasons and therefore the bark had to increase enormously in area to adjust itself to the unusual increase in wood growth. The field observations on fruit trees also seem to show that bark on tree-trunks which increase unusually in diameter during one summer is most

subject to winter-injury while that on those growing at a more moderate rate may be practically immune.

Further observations on apple trees experimentally injured by low temperature.— The three remaining trees which had been treated with a freezing mixture on September 14, 1911, in a seedling orchard at Geneva, were re-examined on June 3, 1912, and found to have changed much in appearance. (See page 32.) Tree 1/25 had sparse and undersized leaves which were of normal color. The tree bore a few small fruits. Many of the distal portions of branches and numerous small spurs had died back and had large numbers of pycnidia of Cytospora broken through the periderm. The branches and leaf area had apparently been reduced by a shortage in the water supply.

Circular " pit-cankers " of various sizes surrounded the numerous " shot-holes " which were made by a bark beetle (Scolytus) a few days after the trees had been subjected to low temperature. The dead pits varied from about 4 to 14 mm. in diameter and most of them had been delimited from the live tissues around them by newly formed cork layers. However, quite a number of the largest ones on the stem and main branches and many of those on the abaxile side at the bases of twigs and spurs had failed to form new cork layers between the dying bark and the wood, and thus resulted in the formation of small circular pits circumscribed by a fissure as shown in figure 8 on Plate XXIII.

The remaining bark on the basal part of the stem had died and the wood underneath had become discolored nearly to the pith. The other wood in the girdled region had a normal color to within about 5 mm. of its outer surface. A thin callus had formed along the upper edge of the girdle.

Tree 2/9 was nearly dead; practically none of the last year's buds had leafed out although the branches had died back only about half way to the main crotches. Numerous small adventitious shoots had arisen on the living portions of the stem and main branches. The leaves on these shoots had a normal color but they were much curled and distorted by aphides.

The pit cankers were more commonly of the larger type and extended to the wood, although there had been sufficient growth to cause their delimitation from the surrounding bark by fissures.

The remaining bark around the base of the trunk had died and the wood underneath it was not only stained to the pith but partially decayed and permeated by the mycelium of some fungus. The other portions of wood had a normal color to within about a centimeter of the exposed outer surface. Practically no callus had developed along the upper limit of the dead girdle. Strips of bark from 1 to 5 cm. wide along both sides of the slits made before the tree was frozen, were partially loose and dead, with numerous pycnidia of

Cytospora bursting through its periderm. The same fungus was also fruiting on the dead portions of the branches and twigs

No additional bark had died at the basal end of the trunk of tree 5/6, and a thick callus had formed around the old wound. The tree had a normal appearance and seemed to be growing nearly as well as the untreated trees around it.

The pits in the bark around the beetle holes were very small and shallow. They had been delimited by cork layers and none were found to reach the wood.

Some additional observations in the Clyde orchards.— The orchards which were discussed on pages 21–23 were visited again on June 18, and August 7, 1912, and found in good growing condition. The callus growths around the winter-injured places of the trees which had had veneer protectors around the trunks had crowded the grafting wax towards the center of the wound and appeared normal.

In the other orchards the growth of callus around the injured regions had also been considerable, as may be seen in figure C of Plate V. The roll of callus surrounding the wound is thick and normal, and although the tree had been more than half girdled it looks like its uninjured neighbors. As far as could be judged by the removal of a small portion of the wax, no rot organisms had entered the dead wood. It appears as though even such severe wounds may be wholly covered on small trees in the course of three years.

The sprouts which had grown from stumps of completely girdled trees were not as promising as it was thought they would be; it would probably have been better to replace them by new trees

A few trees in this orchard were injured during the winter of 1911–12, but on none of them was the bark cracked open. They were more typically canker-like injuries. On the southwest side of several of the Baldwin trees growth had been practically negligible over certain irregular areas, usually of considerable length, while on other parts of the trunk it had been considerable. The place of transition from the normal to areas of negligible growth was distinctly marked by lines, as shown in figure B on Plate V. The area of no growth or the depressed region in this case, extended from near the ground almost up to the first branches, and was broadest but least marked at the crown of the tree. The "sunken" bark still had a green color *externally* and contained much live tissue on June 18. It was found to have some discoloration in the phloem region but much more in the inner cortex. This tree had not been visibly affected during the summer of 1911. On August 7 the entire outer surface of the depressed region was brown and further growth had made the fissure wider than it appeared in the above figure. By removing the dead portions of bark it was found that the injury had extended only to the phloem in most cases and that only here and

there were dead spots as deep as the wood. Cork layers had delimited the dead from the living parts of bark.

Small patches of internally injured bark were also found in some crotches of this and various other trees in the same orchard. Many of the upright shoots which so commonly originate from the larger branches on closely pruned trees, also had the phloem more or less disorganized and the wood slightly stained about their bases, although no trace of the injury could be detected before most of the cortex had been removed. The histological features of such injuries and the changes occurring in them during spring are very interesting and will be discussed in another paper.

Further observations in a Weedsport orchard.— When seen again on July 23, 1912, the injured Baldwin trees in the Weedsport orchard described on page 18, and even those which had appeared uninjured, had not grown very well. They all looked decidedly scrubby and stunted. Only a few of the sprouted stumps had been left and several of them had been "winter-killed." Nearly all of the stumps had been replaced by new trees which seemed to be growing nicely. The Ben Davis trees, however, had grown remarkably well, as may be seen in figure B on Plate VII which is taken down a diagonal row where all the Baldwin stumps had been replaced by new trees.

Since the injured Baldwin trees had not grown at a normal rate the callus growths were also smaller and had made less progress in the process of covering the exposed wood. No additional injuries had apparently occurred in the callus growths of trees injured in winter of 1910–11, nor was any found on other trees of either the Baldwin or Ben Davis varieties.

GENERAL CONSIDERATIONS AND DISCUSSION.

THE CAUSES OF CROWN-ROT.

Introductory.— Although the foregoing observations go to show that initial injuries which eventually result in crown-rot and canker occur *in winter,* and that certain environmental factors and conditions of trees at the close of a vegetative season are in some way related to the occurrence of the diseases, they afford only circumstantial evidence as to the factors or forces actually causing the injuries and the disintegration and rot which follow.

The bark of trees may be injured artificially in various and sundry ways and still give rise to results that may be very similar to each other and to some occurring in nature, but that after all can only be suggestive. If the factors of the environment are not thoroughly studied and sifted to assist in the *selection* of the causal ones, the significant factors actually operative in nature in the production of a disease under consideration may be overlooked. In an endeavor to explain the natural phenomena by means of an agent assumed to be the cause, similar results may often be artificially secured. For

example, gummosis may be produced in various ways, but that does not show that in *nature* it is actually due to *any* of the agents that may have been used to induce it artificially. The first requisite in the investigation of a disease of plants is a thorough study of the environment in relation to the life and seasonal history of the host and the selection for experimentation of the most likely environmental factor or factors and conditions of the host that when combined may result in the disease. That the selection of the chief causal factors is often very difficult is attested by the numerous failures reported in endeavors to make *natural* inoculations on plants with what was thought to be the real cause of disease.

Fungi not the first cause of crown-rot.— In a former paper it was shown that crown-rot had been attributed to various causes by different authors; many suggesting winter-injury as the cause and others fungi, etc. The observations recorded in the present paper show that winter-injury is the first cause, or more accurately that the primary injuries *occur during winter*. The fact that fungi nearly always appear on affected areas in the summer following the time of injury, while some bark is still partially alive and sometimes found exuding discolored " sap," has doubtless given rise to the idea that fungi are the cause. But since fungi seem to be confined to dead areas or to dead spots in severely winter-injured areas of bark it seems more logical to hold, at least until the matter can be more definitely determined, that they are only the agents of decay. In the case of the wood-rotting fungi found in connection with this disease a similar conclusion is reached, because the wood they invade is usually only that which had been stained by the after effects of winter-injury and that killed by exposure.

Can alkali be the cause?—The somewhat plausible assumption that crown-rot is due to an excess of alkali[1] in the soil in some sections of the west where vegetation is apparently often killed by alkali, seems rather unlikely in view of the fact that a very similar disease is equally common in regions where alkali is not present; but more especially in view of observations made by Headden[2] in the same alkali sections, which show that the roots of typically crown-rotted trees at some distance away from the " corroded trunk " are usually normal. It would seem that the more delicate peripheral roots and root-hairs of such a tree would be killed by the alkaline soil solution before the tree trunk could be " corroded " at the surface of the ground.

Arsenic from spray mixtures probably has no relation to the disease.— A little more might be said here about arsenic as the causal agent in relation to this disease although much of the pertinent matter was discussed in a former paper.

[1] E. D. Ball. Is arsenical spraying killing our fruit trees? *Jour. Econ. Ent.* 2:142–48. 1909.

[2] W. P. Headden. Arsenical poisoning of fruit trees. Colo. Agrl. Expt. Sta. Bul. 157:1–56. 1910.

It was pointed out before that crown-rot occurs both in sprayed orchards and in those which had *never* been sprayed; that arsenic is a normal constituent of the soil and often occurs in fairly large quantities and is therefore taken up by plants. Attention was also called to the fact that herbaceous plants grow about crown-rotted trees, as is even shown in some photographs used by Headden. Ball and his associates[1] have more recently shown that large quantities of arsenicals used in spray mixtures may be allowed to stand in contact with the bark of apple trees and be poured about their roots without resulting in harmful effects in one season. On the other hand Swingle and Morris[2] have found that some arsenic compounds are more or less injurious when held in contact with wounded bark of apple-tree branches for some time. But some of their methods are objectionable because the excessive moisture and lack of proper aeration may induce hyperplastic growths and thus admit solutions which probably could not have penetrated the normal cork layers of the bark.

When plants absorb salts containing a poisonous element they are not necessarily injured; especially is that true of trees where so much of the unessential matter absorbed is stored in the non-living cells of the wood. For example, copper is one of the most active plant poisons known, so deadly in fact that it is not advisable to use water distilled from copper vessels when making culture solutions, yet copper is absorbed in the soil solution by plants and may even be stored in enormous quantities. In the vicinity of an abandoned copper mine Lehman[3] found that herbaceous plants contained from 83.3 to 560 mg. of metallic copper per kilogram of dry weight, while the different parts of a nearby cherry tree contained from 8.75 to 112.5 mg. per kilogram of dry weight.

MacDougal[4] also notes the presence of large quantities of *metallic* copper in wood and other cells of *Quercus macrocarpa*.

The chief evidence that has been advanced to show that arsenic causes crown-rot is the fact of its presence in such trees. But as arsenic is also present in normal trees and other vegetation that evidence is worthless; especially when it is borne in mind that trees may store large quantities of poisonous substances without being injured.

[1] E. D. Ball, E. G. Titus, and J. E. Greaves. The season's work on arsenical poisoning of fruit trees.
Jour. Econ. Ent. 3:187–97. 1910.

[2] D. B. Swingle and H. E. Morris. A preliminary report on the effects of arsenical compounds upon apple trees.
Phytopath. 1:79–93. 1911.

[3] K. B. Lehman. Der Kupfergehalt von Pflanzen und Thieren in kupferreichen Gegenden.
Arch. Hyg. 27:1–17. 1896.

[4] D. T. MacDougal. Copper in plants.
Bot. Gaz. 27:68–69. 1899.

Low temperature and excessive or late growth as factors in production of crown-rot.—As stated above these factors have not been experimentally demonstrated as causes of the disease but their causal relation was chiefly inferred from observations made in a large number of affected orchards. The observed facts show that the initial injuries which result in crown-rot occur in winter and that rapidly grown trees are most subject to the disease; they indicate that bark in certain stages of its life history is more susceptible to the injury than in others, and also that the increase in diameter of a tree trunk in relation to its former diameter, as well as the premature checking of bark growth, probably have a causal relation to the occurrence of the injuries.

According to Göppert[1] bark on trees and shrubs is cleft by the drying out of winter-injured bark after thawing and not by the freezing of abundant sap as is commonly held; and that clefts in crotches are caused by the wind while the tissues are frozen and brittle. These injuries are said to be especially common on Prunus and Pyrus.

He found that frequently injuries occurred in the medullary rays and presumably in the inner phloem for he stated that in case the affected parts survive, discolored tissues which are subsequently covered by new annual rings, mark the year of injury. From his observations and remarks made about those of several earlier investigators, it seems as though he had in mind appearances like those shown on Plate VI of the above paper on crown-rot, and the thin discolored line on the left side of Plate XIV and figures 1, 6 and 7 on Plates XXII and XXIII of the present paper.

Göppert held that plants are not susceptible to winter-injury because they contain a superabundance of sap but on account of their stage of development or state of vitality.

Some of the older observations and experiments on this phase of the subject are very interesting. They are to be found in both horticultural and botanical literature. A few of the more pertinent ones, with the conclusions reached, are worth noting on account of the light they throw on the above field observations and because of the diversity of opinion regarding factors involved in the production of winter injuries.

W. H. de Vriese[2] in a discussion on "Some principles of vegetable physiology, bearing on the culture of plants" attributes clefts in trees to the absorbing action of roots in winter: "the rising fluid ascends in trunks of trees and often causes large trees, the expansion of which is prevented by the cold, to split from top to bottom."

[1] H. R. Göppert. Ueber die Wärme-Entwicklung in den Pflanzen, deren Gefrieren und die Schutzmittel gegen dasselbe. pp. XVI+273. 1830. Breslau.

[2] W. H. de Vriese. Some principles of vegetable physiology, bearing on the culture of plants.
Gard. Chron. Agrl. Gaz. 1854:597. 1854.

During the cold weather of January and February, 1855, numerous low-temperature clefts in trees in and around Berlin reopened, and Caspary[1] made a study of the forces concerned in their production.

Early winter had been mild and rainy, but about the middle of January severe cold weather began and the temperature continued below freezing with an occasional freezing rain, until the latter part of February. Measurements of many trees and their clefts were made and the wind and weather records were closely followed through the cold period.

Caspary concluded that clefts in tree trunks occur without reference to the points of the compass and that Göppert's view concerning the relation of the wind as a causal factor can therefore not be correct. He observed, however, that only trees along roads and about the edge of forests are cleft while those in the interior of forests were not cleft.

Since it had been shown by others that after ice is once formed it contracts on a further lowering of the temperature, Caspary maintained that clefts occur in tree trunks as a result of the contraction of the wood, and not because of expansion resulting from ice formation in their interior as many believed. If clefts were formed owing to expansion resulting from ice formation they would close again when the temperature sinks below the freezing point of tree-trunks because ice has a very high coefficient of contraction, but as a matter of fact they open wider.

In a final summary he states that clefts result in introduced annual plants and shrubs during the first severe frost of a season, and that they are caused by the expansion of the abundant sap while freezing, especially in the cambial region; and that such clefts may occur on perfectly normal plants. However, in native trees clefts occur at lower temperatures and are said to be chiefly due to the excessive contraction of the peripheral wood, although in case of large trees in part to the differences in the temperature between the interior and exterior of the trunks. The rupture is said always to occur at the weakest point, determined by the location of decayed parts or wounds.

According to de Jonghe[2] clefts are partially due to sudden changes of temperature in spring which are said to " cause a reflux of the ascending sap," but chiefly to " the sun's rays which cause the bursting of the bark and occasion the splitting." He says that the " rents are always on the side next the sun and never on the east, north, or northwest sides." He held also that " In general, sun-strokes are more common on trees growing in a strong, moist soil, than in one that is light and dry."

In a second article, first published in the Echo de Bruxelles, de Jonghe[3] makes some further additions to his former observations.

[1] R. Caspary. Ueber Frostspalten.
Bot. Zeit. 13:449–62; 473–82; 489–500. 1855.

[2] de Jonghe. Sun strokes.
Gard. Chron. Agrl. Gaz. 1856:213. 1856.

[3] de Jonghe. The sun-strokes in pear trees.
Gard. Chron. Agrl. Gaz. 1856:230. 1856.

He says that sun-stroke is the most destructive disease of pear trees. "The tender, smooth bark of a growing pear tree, being not yet hardened, when struck directly by the solar rays, separates longitudinally from the alburnum to the extent of from two to five inches. The bark cracks in the middle, and its edges curling up, it affords a refuge for insects, which take up their quarters there and contribute by their biting to increase the size of the wound, and to produce a canker which most frequently causes the destruction of the tree." He advocates the covering of the tree stems to protect them from the sun's rays, as is also done by some of our modern horticulturists.

Caspary[1] quotes de Jonghe's last article in full and calls attention to some cases of low-temperature injuries which result in the separation of the bark from the wood, but attributes them to sudden freezing of the sap in the cambial region in spring.

In a further contribution to the cause of wood clefts in trees Caspary[2] confirmed his earlier findings in part by experiment. He found that the width of the clefts is proportional to the degree of cold; and that clefts in smaller trees will open quicker when the temperature becomes low, and close sooner when the temperature rises than those in larger ones. He also noted that old clefts reopen when the temperature is only a few degrees below freezing while new clefts do not form until a considerably lower temperature is reached.

The circumferences of short disk-like sections of tree trunks were carefully measured and then after some of them had been cut radially from the bark to the center with a saw, they were exposed to different temperatures and remeasured. The radial saw cuts which had closed on removing the saw were found open when the temperature had sunk a little below freezing, and two uncut sections were cleft over night by a temperature of — 7.2° C.

Measurements showed that the contraction and expansion of these sections as well as the opening and closing of the radial clefts followed the temperature changes just as the dimensions of the circumferences and clefts did in trees: when the temperature rose their circumferences increased and the clefts closed, while lower temperature decreased the circumferences and opened the clefts. He also found that the circumference of the bark changed more rapidly than that of the wood cylinder.

From these results Caspary concludes that the coefficient of expansion of tree trunks is even greater than that of ice, zinc or iron; and, that trees are cleft because their circumferences decrease more rapidly than their radii when the temperature is lowered.

[1] R. Caspary. Bewirkt die Sonne Risse in Rinde und Holz der Bäume? *Bot. Zeit.* **15**:153–56. 1857.

[2] R. Caspary. Neue Untersuchungen über Frostspalten. *Bot. Zeit.* **15**:329–35; 345–50; 361–71. 1857.

Nördlinger[1] discusses some of the effects of a cold snap which occurred during the first week in September, 1877; he finds that the bark of many young trees was injured and on others shoots were killed back. In the spring of 1878 fungi appeared on the killed bark and the shoots. Injuries were evident in spring as brownish red spots, especially around the bases of twigs and spurs. Some shoots were discolored only on the sunward sides.

Nördlinger concludes that vegetative activities must continue later at the bases of shoots and twigs, perhaps owing to the presence of the food materials usually stored in such places. Most of the shoots that had been severely injured at their bases subsequently died. On older structures the injurious effects were usually proportional to the size of the injured areas.

He holds that R. Hartig's contention that such crotch-cankers are due to spring frosts is erroneous, as may be seen by the examination of cross-sections and also on account of the fact that thousands of cases occur high up in trees and in locations where spring frosts could not have been harmful. He thinks it more likely that the tissues of such injured places entered winter in an immature condition and were injured on that account.

In a brief note in the Gardener's Chronicle signed by A. D.[2] it is stated that in December, 1887, a few old plants of Japanese chrysanthemum were found having the bark loosened on their stems, above the ground. The growth of the plants had been checked by drought and they grew again in fall. The bark was thought to have been loosened by hoarfrost fractures.

The measurements of tree trunks made at different temperatures during last winter and spring confirm Caspary's observations in showing that the lowering of temperature decreases and the raising of temperature increases the circumference considerably. From a few experiments done last winter with cross-section of large maple branches and trunks of apple trees it was found that the circumference of isolated rings of bark decreases appreciably more than that of the cylinders of wood when subjected to the same low temperature and would thus apparently lead to an increase in bark tension.

It is a well known fact first clearly set forth by Sachs in his Experimental Physiologie that tensions between different kinds of tissues result from differences in their rates of growth. The existence of tensions between the bark and wood of a fruit tree can be readily demonstrated by slitting the bark. This method may be used to show that there is often quite a variation in bark tension of different trees as well as at different heights on the same tree.

[1] Nördlinger. Die September-Fröste 1877 und der Astwurzelschaden (Astwurzelkrebs) an Bäumen.
Centbl. Gesam. Forstw. 4:489–90. 1878.

[2] A. D. Effects of recent frosts.
Gard. Chron. 2:691. 1887.

Kraus[1] measured at various heights the bark tensions of many trees, shrubs and herbaceous plants by isolating rings of bark and replacing them to find the amount of contraction that had resulted. Although the method gives neither quantitative nor even accurate comparative results, it shows beyond question that there are regions of maximal and minimal transverse bark tension at different heights on a trunk.

In a one-year-old shoot or stem the transverse tension between the bark and wood was found to increase from the tip downward so that the basal internode had the greatest bark tension. As growth continues the tension increases up to a certain point and then the bark cells divide and grow, and thereby reduce the tension until further growth increases it again. This process is said to begin at the oldest internodes and to pass gradually towards the distal end.

In some measurements of transverse tension taken during winter considerable variation was found in the distribution of maxima and minima on the stems and branches of trees, but it usually began with zero at the distal end and increased to the maximum on the largest branches before reaching the main crotches and afterwards increased to a second maximum somewhere about the crotches. In some instances the bark tension of stems decreased towards the ground and in others it was found to increase to another maximum at the surface of the ground or about the upper roots. Kraus holds that transverse tension develops in the bark and gradually increases to a maximum because the bark lags more and more in growth until at a certain stage in its life history its outer portion ceases growth and is ruptured, resulting in the roughened bark typical for the species. The cortical parenchyma seems to be tardiest in tangential growth during the first few years and therefore suffers the greatest transverse or tangential strain, until after a certain number of years it reaches a maximum which is followed by a more rapid growth and a consequent reduction of the tension.

It is thus seen that transverse bark tension has seasonal and life history maxima and minima which may change their positions up and down tree trunks and branches. It was also found that there is a daily periodicity in the tension with a maximum at night and a minimum about 2 P. M. It is held that the daily periodicity cannot be due to root pressure nor to differences in transpiration because cut branches immersed in water still exhibited the same periodicity. Neither do temperature variations appear to affect the daily periodicity as long as they do not go below vegetative requirements. But when a branch was placed in the dark about noon (the period of minimum tension) the maximum was reached in one or two hours and remained so till exposed to light, when the normal periodicity was again resumed.

[1] G. Kraus. Die Gewebespannung des Stammes und ihre Folgen. *Bot. Zeit.* 25:105–19; 121–26; 129–33; 137–42. 1867.

Swaying is said to decrease transverse tension at the point of bending and is followed by an increase in the rate of growth at that region.

Kraus also found many very small longitudinal clefts in the smooth outer bark of Acer, Æsculus, and Salix, during winter and extending as far as the cambium in some cases. The clefts are attributed to transverse tension.

From this work it appears highly probable that toward the end of a vegetative season the bark tension on a tree which increased much in diameter as compared with its former diameter, may be much greater than on one having made but little growth. It is also apparent that in case vegetative activities are inhibited prematurely in fall or continued in full vigor abnormally late owing to uncommon environmental conditions, the seasonal maximum bark tension may be retained through the following winter. The bark on trees entering the dormant season with high growth tension maxima at certain regions of trunks and branches, is subject to excessive strains in the high pressure areas when the temperature drops suddenly through many degrees; that would be true regardless of whether the temperature sank low enough to be injurious to perennial plants or not.

The above field observations show that the initial injuries resulting in crown-rot and cankers usually occur at points which are in close agreement with the location of the regions of maximal bark tension found by Kraus, and in view of the researches of Sorauer which seem to show that low temperature injury is often due to the tensions induced rather than to the degree of cold, it appears probable that initial injuries of this type are due to the combined effect of tensions resulting from differences in growth rate, their increase by low temperature, and the additional strain caused by bending in time of strong winds.

In the case of the experiment discussed on pages 32 and 44, the temperature was probably too low to enable one to distinguish between the effect of the degree of cold and the tensions induced. It seemed strange, however, that some bark was loosened on a tree on which it had been slit; yet it does not show that the old notion of slitting the bark on the trunks of rapidly growing trees is erroneous, for it may be that if slit at certain times and allowed only time enough to heal the wounds before the dormant period arrives some injuries due to excessive bark tensions might be avoided.

The wind as a factor in causing crown-rot.— As was often noted in the field observations the relative wind exposure to which different orchards are subjected seemed to make a decided difference in the amount of winter-injury resulting. The injuries occurring on tree trunks above ground and below the crotches were usually oriented with reference to the prevailing wind. In case the location of the injuries was not determined by the prevailing wind they were nevertheless on the *same side* in any particular orchard or locality, indicating that the wind may have come from that direction during the

time the injury occurred. It appears significant also that in instances where trees had been banked with soil the injuries occurred just above the soil while on unbanked trees it usually occurred at the surface of the ground. But this probably does not hold when the ground is not frozen at the time of injury for in that case any bending that may result from strong winds would probably occur at the surface of the ground or even below it, and result in injury at the root crotches.

Göppert[1] maintained that the wind is involved in the production of winter injuries, especially the north wind, for injuries were sometimes found confined to plants in certain strips or zones of localities. Excessive evaporation, an additional lowering of the temperature due to wind he believed, were frequently the causes of killing back of shoots and branches of trees.

Bernbeck[2] subjected various plants to wind rates as high as 31.3 miles per hour and obtained some very striking results. He found that transpiration was proportional to the wind rate and to the amount of bending or swaying undergone by plant structures, and that the excessive loss of water ceased when bending was eliminated. His results with lignified plant structures are especially pertinent to this discussion.

A potted plant of *Fagus silvatica* was exposed to a wind rate of 31.3 miles per hour (14 m. per second) and in about 10 days brown spots had developed in the bark, where both the cambium and cortical perenchyma had been killed. Many cell walls are said to have been ruptured. On a potted plant of *Picea excelsa* exposed to the same wind rate a few hours, the bark on the swaying twigs was cleft open at a number of places on the windward or convex side, exposing the bare wood. *Pinus silvestris* and *P. austriaca* sustained similar wounds on the convex sides of twigs when subjected to the same treatment. The clefts closed and became invisible when the normal environment was restored. *Ulmus effusa* was also injuriously affected, but in this case the uninjured areas of bark remained alive, and after a few weeks the damage done to the woody parts had been practically repaired by growth.

On a potted specimen of *Alnus incana* given the same treatment, the bark was injured in many places after several days' exposure. Some injuries occurred also in the wood cylinder but were not evident externally. Knotty masses of callus developed on the injured regions. *Quercus pedunculata* was similarly affected after 6 days' exposure and also developed knotty enlargements about the old wounds in the course of the summer. Thin lignified twigs of *Ulmus effusa*, *Alnus glutinosa*, *Fagus silvatica*, *Picea excelsa*, and *Larix europaea* were exposed in duplicate (one set being firmly fixed and the other allowed

[1] *L. c.* pp. 58–61.

[2] O. Bernbeck. Der Wind als pflanzenpathologischer Faktor. Inaug. Dissertation, Bonn. p. 116. 1907.

to sway) to an air current of 22.3 miles per hour (10 m. per second), and after 3 to 6 days all of the loose twigs had died back more or less while the fixed ones remained apparently normal to the end of the experiment. That is, perfect rigidity seems to afford total immunity from wind injuries, while swaying or bending result in injury.

In order to distinguish between the swaying and drying effects of strong winds the experiments were repeated, the plants being kept wet by means of a spray of water. Similar results were obtained, and it was found that the bending of plant structures has a marked influence on the transpiration rate of both leaves and twigs even when the surrounding air is saturated with moisture.

When the wind rates used in these experiments are compared to those frequently occurring during the dormant period of fruit trees they are not very high, and their pressure is therefore also lower than that to which our trees are often subjected. The following table taken from an article by Keller[1] shows the pressure resulting from different wind rates.

TABLE IV.—THE RATE AND RESULTING PRESSURE OF THE WIND.

DESIGNATION.	Miles per hour.	Pressure per sq. ft.	DESIGNATION.	Miles per hour.	Pressure per sq. ft.
Fair breeze	5	0.126	Stiff breeze	18	1.634
	6	0.181		19	1.821
	7	0.247		20	2.018
Fresh breeze	8	0.323	Very brisk wind	25	3.155
	9	0.408	High wind	30	4.547
	10	0.505		35	6.194
	11	0.610	Very high wind	40	8.099
	12	0.726	Gale	45	10.260
	13	0.852	Storm	50	12.684
	14	0.988	Great storm	60	18.310
Stiff breeze	15	1.135	Hurricane	80	32.800
	16	1.291	Tornado	90	40.500
	17	1.458		100	50.000

A wind blowing at the rate of 25 to 40 miles per hour is not at all uncommon in most regions of the United States and frequently a rate of 50 to 60 miles is maintained for some time.

Bernbeck's results are especially interesting when compared with the above field observations of 1911. Most of the injuries occurring during the winter of 1910–11 were accompanied by radial clefts in

[1] E. Keller. The hygiene of the small chemical laboratory. *Jour. Ind. Eng. Chem.* 2:246–51. 1910.

the bark at the region of severest injury, and the bark was loosened on the windward and sometimes also on the leeward side as shown on Plate I. The wood was not cleft, and sometimes only the periderm and outer cortex were cleft and loosened. The trees had not been injured in extreme cold weather, because during that winter the injury occurred before the middle of January. However, the cases observed near Glens Falls were slightly different in that the injury involved a longer portion of the trunks and was sometimes confined to the region nearly midway between the ground and first crotches, as shown in figure B on Plate VIII. It seems as though the bending might have occurred higher up or over a greater length than it did in the western part of the State, possibly due to the frozen condition of the trees and ground at the time of injury. Since the location of the region of maximal bark-tension may determine the place of injury, it appears possible that the tensions were more widely distributed in this case.

When a late summer drought retards bark growth or its complete adjustment to the increased circumference of the new wood produced in early summer, smooth bark may not only have a high transverse tension when the winter or dormant season begins but its lenticels may also be in such a state of incompleteness as to permit excessive evaporation throughout the following winter, and especially during a windy winter thaw. The bark on trees in wet situations may be in a similar condition on the approach of the dormant season, owing to abnormally late growth of wood in mid-summer. In view of Bernbeck's observations and experiments which show that the bending of twigs and branches by the wind results in excessive loss of water and consequent injuries on the windward side of shoots, it is very likely that imperfect lenticels and a high bark tension increase the liability to such injuries. The type of bark injuries which occurred on smooth-barked, thrifty pear trees in the winter of 1910–11, and which resulted in much blackened bark on the west side of trees as described above for some Medina orchards, seems to be of this type. During the winter of 1909–10 a similar though less extensive injury developed on the south side of young pear, apple, plum and cherry tree trunks and ascending branches in the western part of this State; while in the Hudson River Valley about Poughkeepsie and Milton, the injury occurred on the north side of trees during the same winter. In the winter of 1909–10 as well as in that of 1910–11, partial or complete winter thaws occurred during strong winds. In the former the wind was very high from the south during several days of open weather in late winter in the western part of the State, and in the latter the wind was from the west in the same region during a January thaw. Such injuries are usually called "sun-scald" and are attributed to an injuriously high temperature induced in such bark by the sun, but since the injuries may also occur on the north

side of trees and on trees so situated that the sun rarely shines on their trunks the " sun-scald " hypothesis appears untenable, while the wind swaying and excessive evaporation theory of Bernbeck seems to afford an explanation of the observed facts. As stated before, more or less thickly scattered groups of cells in the inner phloem and inner cortex are injured first and sometimes all around a trunk or an ascending branch, but in severe cases the interspersed living groups also die; first on the windward side, and in extreme cases the entire bark subsequently dies on upright shoots. Such shoots are then said to have been " winter-killed." In the " sun-scald " and " winter-killed " types of injuries the bark does not sustain radial clefts but is frequently wind-checked after it dies. In case the initial injury in the phloem and inner cortex has not been severe enough to result in the typical " sun-scald " effect the bark on the windward side of trunks frequently dies only in patches during the summer, as is shown in figure C on Plate XII.

The injuries of last winter which included radial clefts, usually also involved the wood cylinder. In many of these cases the bark was not loosened. In considering the contraction of tree trunks (page 36) occurring during the time of injury it seems likely that the clefts, especially in cases where the bark had not been loosened, were mainly due to low-temperature contraction in the wood. Yet it appears probable that wind swaying was also concerned in the production of wood clefts which extended *through the entire wood cylinder* like those shown on Plates XIV and XXIII, because it is inconceivable how tensions due simply to peripheral contraction could result in wood clefts extending far beyond the pith. In instances like the one shown on Plates XXII and XIII, where there was a combination of bark loosening and radial clefts in both wood and bark, the bark tension must have been high on the approach of winter. It would appear, then, that the chief difference between the winter injuries of the past two years involving radial clefts, is their occurrence at different temperatures. In the winter of 1910–11 the injuries occurred during only moderately low temperature and the bark alone was cleft; while last winter the temperature was very low during the time of their occurrence, and resulted in wood clefts also. In many cases of last winter the bark loosening did not accompany the clefts, presumably because the tension in the bark was but little in excess of that in the wood. But the wind was probably a factor in both cases.

Low temperature probably caused the bark injuries in crotches of young apple trees by increasing the bark tension due to growth. The wind seems to have had only a secondary influence in those cases since no clefts resulted. Nördlinger attributed crotch injuries to early cold weather and the immature condition of the tissues at such points.

The relation of growth and low-temperature tensions and the strain induced by high winds, to the initial injuries resulting in rots

and cankers on fruit and shade trees has been inferred chiefly from the environment of affected trees and from the type and location of the effect produced on them. The crown, "heart," and root rots which follow are doubtless due to fungi, principally of the hymenomycetous group.

SOME SUGGESTIONS OF ECONOMIC BEARING.

The field observations indicate that varieties of fruit trees which are subject to winter injuries of this type should be headed low regardless of the inconveniences which may be experienced in cultivation; also, that excessive and late growth should be prevented if possible. Perhaps windbreaks or some means to prevent young trees from swaying, may also prove of value in preventing the initial injuries. *Young trees which are growing rapidly or trees whose growth was prematurely checked by unfavorable conditions, so that they enter the dormant period with immature bark, ought to be carefully examined in spring and early summer for indications of loosened or injured bark. Such bark when found should be cut out with a sharp knife at right angles to its surface and the exposed wood, if still of normal color, covered with grafting wax or with tar paint if it is discolored or dead.*

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THE APPLE AND CHERRY ERMINE MOTHS

P. J. PARROTT AND W. J. SCHOENE

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TECHNICAL BULLETIN No. 24.

THE APPLE AND CHERRY ERMINE MOTHS.

P. J. PARROTT AND W. J. SCHOENE.

SUMMARY.

During recent years colonies of the caterpillars of the apple and cherry ermine moths have been discovered in considerable numbers in the State of New York. These insects were introduced in shipments of foreign nursery stock and appeared in plantations of imported apple and cherry seedlings. According to the records of the Division of Nursery Inspection infested plants have been found at Lockport, Hilton, Chili, Dansville, Rochester, Penfield, Newark, Orleans, Seneca and Geneva in western New York; at Johnstown and Schoharie in the Mohawk Valley region, and at Blauvelt, in the Hudson River Valley.

From the material that has been collected two species of moths were bred — *Yponomeuta malinellus* Zell., which thrives largely on apple, and *Y. padellus* L., which is a more general feeder, showing preference for hawthorn, plum and cherry. Both species are common and destructive fruit pests in Europe.

The adult insects are small moths, with snowy white, black-dotted anterior wings. The hind wings are gray or leaden in color, with long fringes on lateral and posterior margins. The wing expanse is about 20 mm. The caterpillars are quite variable in color, ranging from pale to grayish or greenish brown, and they average about 15 mm. in length. They have web-forming habits and live in a common web, and in this they spin their cocoons.

In the studies on the life history of these insects during the past four years the moths appeared during the first two weeks in July, and oviposition began about the middle of this month. The eggs are deposited in oval-shaped masses near a bud, usually of the current year's terminal growth, and less frequently on the older wood. Hatching takes place in early autumn and the young larvæ remain through the winter under the protecting crust of the egg shells. In the spring they assemble among the tender leaflets of an adjacent bud, which they attack. The older caterpillars feed openly on the foliage under the protection of a thin, grayish web. With the need of more food they extend their webs, seizing and involving fresh leaves in a common nest. In severe attacks trees may be defoliated and completely covered with the silken tents of the insects. Pupation took place during the latter part of June and early July and the moths lived from the beginning of July to about the middle of August.

These insects have, in their normal habitat, a large number of natural enemies, the most important of which belong to the orders Hymenoptera and Diptera. In spite of the large numbers of the moths' eggs imported into the United States, the lepidopterons were apparently unaccompanied by their more common and efficient parasites. An ichneumon, *Mesochorus* sp., was obtained from *padellus* reared on cherry, and a tachinid, *Exorista arvicola* Meigen, was quite abundant in some colonies of *malinellus* caterpillars subsisting on apple.

Comparisons of the structures of the caterpillars and of the male genitalia show no tangible structural differences between *padellus* and *malinellus*. The absence of differential features suggests that the moths from hawthorn and cherry and those from apple constitute a single species; but cross breeding experiments are desirable to settle definitely the status of the two forms.

An outbreak of these insects is to be expected from two sources: (1) From the annual importation of infested foreign-grown nursery stock, and (2) from spread of the pests that may have established themselves along the avenues of trade in previous shipments. The remedy is careful inspection of nurseries during June and the destruction of infested plants. As fruit pests, the insects would prove amenable to prevailing spraying practices.

INTRODUCTION.

A study of the insect outbreaks from year to year in the State of New York will impress one with the number of introduced species and their great importance to its fruit interests. These constitute a steady and a severe drain on its horticultural resources. Many of the principal introductions in the past had their origin in Europe, and in the diverse and constantly increasing intercourse with the United States there is a marked trend of migration of the common and destructive species to this country. Common pests of fruit trees in all parts of the continent are certain insects, known as "ermine moths," which are discussed in almost every leading work upon European economic entomology. Interest is now directed to these insects as they have during recent years been brought into this State in considerable numbers in foreign importations of nursery stock. It is desired to call attention to their injurious nature, the circumstances of their discovery and the danger that exists of these pests being introduced, if they have not already become established.

THE ERMINE MOTHS.

GENERAL CHARACTERS.

These moths constitute the genus *Yponomeuta* of the family *Yponomeutidæ*. In Dyar's list of North American Lepidoptera this

family is placed between the Tortricidæ and the Gelechiidæ. The genus is a small one, but it contains a few species which, because of their common occurrence and economic importance, are well-known insects in their normal range of distribution. The moths are small and have an expanse of wings which varies from about twenty to twenty-five millimeters, according to the species. A characteristic feature of these insects is that the anterior wings of most species are brilliantly snowy white, and marked with black dots. The hind wings are generally darker, being grayish or leaden in color, and possess long fringes on the lateral and posterior margins. The caterpillars are gregarious and have web-forming habits. They live in a common web which may involve many twigs and leaves, and in this they spin their cocoons.

HISTORICAL NOTES AND SYNONYMY.

The history and synonymy of *padellus* and *malinellus* are as follows: The former was described by Linnæus [1] in 1758 under the name of *Phalæna (Tinea) padella*,[2] and fruit trees are given as its host plants. Fabricius [3] in 1775 describes the moth, larva and pupa and states that the insect occurs on fruit trees. Believing that it was distinct from *padellus*, Zeller,[4] in 1844, designated the form occurring on apple as *malinellus*. The species described by him as *variabilis* is listed by most writers as a synonym of *padellus*.

Latreille [5] in 1796 describes the genus *Yponomeuta*,[6] and in 1802 [7] he gives *Tinea evonymella* L. as the type. Stephens[8] in 1829 established the family *Yponomeutidæ*. Sodoffsky[9] in 1837 changed

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[1] Linnaeus. Systema Naturæ, 10th Ed. 1: 535. 1758.

[2] *Padella*, so named from *Prunus padus* L., the European bird cherry. The Linnæan names for several of the *Yponomeuta* species are very misleading. *Prunus padus* is the principal host plant of *Y. evonymellus* L., but the insect's name would imply that it lives on Evonymus. This confusion Zeller sought to correct.

[3] Fabricius. Systema Entomologiæ, p. 656. 1775.

[4] *Isis*, p. 220. 1844.

[5] Latreille. Précis des Caractères génériques des Insectes, p. 146. 1796.

[6] This is of Greek origin ὑπονομεύω "I mine," and is presumably taken to mean "one who undermines" or "who works under the surface". The derivative ὑπονομεύτης belongs to the first declension masculine, and we have therefore used "us" endings in the names of the species.

It should also be noted that words which begin with "upsilon" have the rough breathing, which is represented by the letter "h" in words derived from the Greek, — thus ὑποκριτής becomes hypocrite in English. If Latreille had followed this rule he would have designated his genus Hyponomeuta instead of Yponomeuta, but in spite of its unusual formation, which is a modern invention, there appears to be no other choice than to accept his selection.

[7] Latreille. Histoire Naturelle générale et particulière des Crustacés et des Insectes. 3: 467. 1802.

[8] Stephens. Catalogue of British Insects, p. 193. 1829.

[9] Sodoffsky. Etymologische Untersuchungen ueber die Gattungsnamen der Schmetterlinge, p. 21. 1837.

Yponomeuta to *Hyponomeuta*[1] which was adopted by Zeller, Stainton and other systematic and economic writers. In Dyar's[2] list, 1902, Busck reverted to the original orthography *Yponomeuta*, which we have adopted.

ATTACKS UPON FRUIT TREES; HOST PLANTS.*

Many of the ermine moths attack fruit trees. The most common and destructive species is perhaps *Yponomeuta padellus* L., which feeds principally on the cultivated plum, blackthorn (*Prunus spinosa* L.) and hawthorn (*Cratægus oxyacantha* L.). Taschenberg,[3] Boisduval,[4] Rebaté [5] and Theobald [6] record it as attacking either wild or cultivated cherries. There has been some doubt as to whether *padellus* actually thrives on apples, but many writers including Major,[7] Delacour,[8] Westwood,[9] Stainton,[10] Köllar [11] and Ormerod [12] list this fruit among its host plants. In England *padellus* has been generally regarded as the species attacking apple. Schöyen [13] of Norway states that *padellus* is the common species on apples, and Bos [14] of Holland has observed *padellus* migrating from *Cratægus* to apple trees. Nördlinger,[15] Mokshetsky [16] and Reh [17] record the pear as one of its hosts. Other plants mentioned by various writers are the medlar (*Mespilus germanica* L.), the European mountain ash (*Sorbus aucuparia* L.) and the ash (*Fraxinus excelsior* L.). In describing conditions in Crimea Mokshetsky [16] gives the white willow (*Salix alba* L.) and the spindle tree (*Evonymus verrucosa* Scop.) as the chief host plants, and the larch and the plum (*Prunus domestica*) as less subject to attack.

[1] See footnote 6 on previous page.
[2] Dyar. North American Lepidoptera, p. 489. 1902.
[3] Quoted from Judeich-Nitsche's Forst-Insektenkunde, **2**: 1069.
[4] Essai sur l'Entomologie Horticole, p. 574. 1867.
[5] La Chenille Fileuse du Prunier, p. 8. 1909.
[6] Insect Pests of Fruit, p. 91. 1909.
[7] Treatise on Insects. 1829.
[8] Essai sur les Insectes, p. 293. 1850.
[9] *The Gardeners Magazine*, **13**: 433–439. 1837.
[10] Lepidoptera Tineina, pp. 58–61. 1854.
[11] Treatise on Insects, trans. by Loudon, p. 226. 1840.
[12] Manual of Injurious Insects, p. 263. 1881.
[13] *Ztschr. Pflanzenkr.*, **3**: 268–269. 1893.
[14] Letter of April 26, 1910.
[15] Die Kleinen Feinde der Landwirthschaft, p. 460. 1869.
[16] The Apple Moth, p. 15. 1907.
[17] Handbuch der Pflanzenkrankheiten (Sorauer), **3**: pp. 271–274. 1909.

* The difficulty of identifying certain of the ermine moths has led to confusion as to the food plants of each. This is especially true of the species *padellus* and *malinellus*, which have unquestionably been mistaken one for the other, and perhaps confounded with other forms of very similar appearance.

Y. malinellus Zell., as the name implies, thrives on the apple, which constitutes its favorite host. Marchal [1] records its occurrence in destructive numbers on the almond (*Amygdalus communis* L.). Dahlbom, according to Kaltenbach,[2] included the wild service tree (*Sorbus torminalis* Crtz.). It has not generally been considered as a British species, but on the other hand Theobald [3] asserts that it exists in that country on apples and has been confused with *padellus*. Kuwana [4] states that while *malinellus* occurs most commonly on apples in Japan, it nevertheless may feed on the sand pear (*Pyrus sinensis* Lindl.), the Chinese flowering apple (*P. spectabilis* Ait.), the quince (*Cydonia vulgaris* Pers.), the peach (*Amygdalus persica* Sieb. and Zucc.), the Japanese flowering cherry (*Prunus pseudocerasus* Lindl.), and the apricot (*Prunus armeniaca* L.).

Besides these two moths there are several other species of relatively minor importance which are recorded as attacking fruit trees; and these are respectively as follows:

Y. mahalebellus Gn., which is recorded by Marchal [1] as common on the Mahaleb cherry.

Y. cognatellus Hb. is very similar in appearance to the foregoing species, and according to Wahl [5] and Kirchner [6] is a common pest on cultivated plums. Mokshetsky [7] records it also upon the Mahaleb cherry and apple.

Y. irrorellus Hb. occurs, according to Hess,[8] principally on the spindle tree (*Evonymus europœus* L.) and sometimes feeds on plum.

Y. rorellus Hb., which is said to be similar to *padellus*, commonly thrives on the willow. Rössler,[9] however, reported its occurrence on cultivated plums.

Y. evonymellus L., which is quite readily distinguished from other species by its size and larger number of black spots on wings, breeds chiefly on the European bird cherry (*Prunus padus* L.). Lunardoni [10] reports it upon cultivated cherries, and Theobald [3] on both cherries and apples.

COMMON NAMES.

As they are quite common insects and are widely distributed throughout Europe, these moths have been designated by many local and popular names: Delacour [11] called *padellus* "La teigne hermine", the "Ermine moth", which was suggested by the anterior

[1] *Bul. Soc. d'Étud. et Vulg. Zool. Agric.*, p. 17. 1902.
[2] From Kaltenbach's Die Pflanzenfeinde, p. 194. 1874.
[3] Insect Pests of Fruit, p. 91. 1909.
[4] Letter of Mar. 31, 1911.
[5] Die Bekämpfung der Gespinstmotten, p. 4. 1907.
[6] Die Obstbaumgespinstmotten, p. 3. 1905.
[7] The Apple Moth, p. 15. 1907.
[8] Die Feinde des Obstbaues, p. 259. 1892.
[9] Quoted from Kaltenbach's Die Pflanzenfeinde, p. 169.
[10] Mentioned by Marchal in *Bul. Soc. d'Étud. et Vulg. Zool. Agric.* 1902, p. 23 (footnote).
[11] Essai sur les Insectes, p. 293. 1850.

silken white wings punctuated by black spots. This name has generally been accepted for popular usage in England, where these moths are known as the "Small",[1] or "Little, ermine moths". Species of economic importance in that country are commonly referred to as the "Hawthorn ermine moth" or the "Apple ermine moth", etc., according to the host. Judeich-Nitsche[2] designated this group of moths as "Schwarzpunktmotten", which obviously was also suggested by the aspect of the front wings. The gauzy texture of the webs or tents spun by the caterpillars doubtless led Jablonowski[3] of Hungary to call these insects "Pókhálós Molyok", "cobweb moths", and in taxonomic treatises by German writers these insects are frequently referred to as the "Gespinstmotten", which again suggests their web-spinning habits.

The more destructive species have received a variety of names throughout their range of distribution. Particularly is this true of the form attacking apples which in England is known as the "Apple ermine moth" (Theobald, Collinge); in France as "La chenille fileuse du pommier" (Rebaté) or "L' hyponomeute du pommier" (Marchal); in Germany and Austria as the "Apfelgespinstmotte" (Wahl, Kirchner, Hess); in Norway as "Eplespindmol" (Schöyen), in Sweden as "Apelspinnmal" (Lampa); in Crimea as "Yablonnaya mol", the "Apple moth" (Mokshetsky); and in Japan as the "Ringo no sumushi", the "Apple veil worm," or "Ringo kemushi", the "Apple caterpillar" (Kuwana). These also indicate the importance which is attached to this species as a destructive pest of the apple.

ECONOMIC IMPORTANCE.

The ermine moths are regarded abroad as very destructive pests of fruit trees, and because of their importance to horticultural interests, standard European works of reference on orchard insects usually contain a very complete account of these species. *Y. padellus* has largely derived its reputation for destructiveness from its attacks on hawthorns and plums, and with these some writers would also include cherries and apples. During certain seasons it is a very common pest on hawthorn which may be rendered very unsightly

[1] The prefix "little" as frequently used by British entomologists in this connection is apparently employed for the purpose of distinguishing these moths from some arctiid species which are of somewhat similar appearance but are much larger. Kappel and Kirby in British and European Butterflies and Moths (1895) mention the following species: Ermine moth (*Spilosoma lubricipeda* Fabr.); Water ermine moth (*Spilosoma urticæ* Esp.) and White ermine moth (*Spilosoma menthastri* W. V.). In this connection it is also of interest to note that Riley in "Shade Trees and their Insect Defoliators", U. S. Dept. Agr. Ent. Bul. 10, referred to the form *punctatissima* of *Hyphantria cunea* Drury as the "Many spotted ermine moth", while Smith in Economic Entomology, p. 266, designates *Spilosoma virginica* as the "White ermine moth".

[2] Forst-Insektenkunde, 2: 1067.

[3] A Gyümölcsfák és a Szóló Kártevó Rovarai, p. 45. 1902.

by being stripped of the foliage while the bare wood is covered by the conspicuous webs or tents of the insects. In the leading plum-growing districts of France, such as the "Départements" of Lot-et-Garonne, Tarn-et-Garonne, Lot, Dordogne and Gironde, this species constitutes one of the chief pests of this fruit. Rebaté and Bernès report that serious outbreaks of the insect occur periodically. In an account of the history of the pest in the Département of Lot-et-Garonne they state that in 1843 all trees were attacked and from 1867 to 1871, in 1882 and again in 1888 much damage was done by it. There was an outbreak in 1901 which was followed by a more severe one in 1902, and it was not until 1904 that its injurious attacks ceased. During 1908 the caterpillars again increased to destructive numbers and serious depredations to plums occurred during 1909.

The apple is especially susceptible to attacks by the ermine moth and wherever this fruit is grown in Europe this insect is one of the most common and destructive pests. In 1838 according to Maurice Girard,[1] "the farmers of Normandy [France] beheld the distressing spectacle of [apple] trees stripped of their foliage and covered with thousands of caterpillars. These, having nothing more on which to subsist hung here and there suspended in enormous masses within a web, while the trunks of the trees were enshrouded with a silken web which concealed the bark. Not only was the crop destroyed in various cantons for several years, but a large number of trees in full bearing succumbed to the injuries." Marchal reports that in certain areas of France *malinellus* appears almost every year in more or less destructive numbers, and that in some communities where there have been serious outbreaks for successive years almond trees have been killed. This species was, in 1902, very abundant and destructive throughout France.

Theobald regards *malinellus* as an important pest of apples in England. According to Whitehead[2] an Yponomeuta caused "exceeding destruction in this country in 1865. It was also very troublesome in most of the large apple-producing districts in the year 1877 and in some few places again in 1880. * * * Whole orchards were entirely devastated in the two first-named years, so that at the commencement of July the trees were as bare of foliage as in December. Leaves, blossoms and fruit were all cleared off by the innumerable caterpillars which not only devoured every particle of these, but also actually began to gnaw the most tender portions of the fruit-bearing spurs. Not only did they utterly ruin the crop in these seasons, but they also injured the trees so extensively that they only yielded a small crop in the subsequent seasons."

Reh[3] writes that *malinellus* was a great scourge in Germany during 1910, and that apple trees were seriously affected.

[1] Quoted from G. Barbut, *Le Progrès Agricole et Viticole*, p. 307. 1899.
[2] Rept. Inj. Ins. to Fruit Crops No. 3, p. 68. 1886.
[3] Letter of June 27, 1910.

Lampa records that in 1908 *malinellus* ravaged apple orchards generally in Onsala and Fjäre in the Province of Holland, Sweden. In one orchard all trees were completely attacked, not a single one escaping. A lesser attack occurred in 1909. During the outbreak of this year the trees were defoliated in five days and were so covered with webs that they had the appearance of a " fur coat ".

According to Mokshetsky *malinellus* has caused much damage to apple orchards, especially in the regions of Russia subject to dry climate. It is a common insect in fruit plantings, but from time to time it increases to incredible numbers. These outbreaks are of a periodical nature and coincide with years of exceptional droughts, approximately once in ten years. Such took place in 1874–75, in 1884–86, in 1894–96, and lastly in 1904–05. Orchards that are severely attacked have the leaves all eaten off, standing as bare as in winter, and are merely covered by a web containing worm-eaten fragments and clusters of the cocoons of the moth. The remnants of the leaves dry up and redden. In consequence of the heat of summer, growth is slow, and under such conditions the production of fruit may be checked for several years. According to Schreiner the yearly loss to the apple crop in the Government of Saratov alone approximates three million marks.

Kuwana says that it is one of the most troublesome insects of apple growers and is a familiar species to most persons in the apple-growing regions of northern Japan. The conspicuous feature of its damage is the defoliation of the trees.

Saracomenos says that a large number of fruit trees, such as apple, pear and plum, which are grown on an extensive scale on the Island of Cyprus, are attacked by *padellus* and *malinellus*. These insects may not only destroy the crops, but if they appear in large numbers for a series of years they may also cause the death of the trees. Other writers in still different fruit-growing regions of Europe comment in like manner on the destructive capacity of these insects.

DISTRIBUTION.

These two moths are generally distributed throughout Europe. The form *padellus* occurs in England, Scotland and Ireland, and on the continent, ranging from Norway and Sweden to the north and Italy and the Island of Cyprus to the south. Staudinger [1] and Rebel mention in its range of distribution Sarajevo, Croatia, Fiume, Dalmatia, Siebenbürgen, Roumania, West Bulgaria, Greece, Armenia and Tura (West Siberia). According to Köppen it probably occurs over a greater part of European Russia and Turkestan.

The associated form, *malinellus*, has a range similar to the foregoing species. Mokshetsky states that in the extreme northwest of Russia and in Poland, where there are many orchards of the

[1] Ann. d. K. K. Naturhis. Hofmus. Wien, 1904, p. 346.

principal varieties of apples, the moth occurs only in trifling numbers; but it is most severely felt in southern and middle Russia where the dry, continental climate of this region seems to be more favorable for the propagation of the insect. Its distribution is largely confined to the area of apple growing, which is bounded by the governmental states of Livonia, St. Petersburg and Viatka on the north to Taurida, Saratov and Kursk southward. Unlike *padellus* this moth occurs in Japan, and according to Kuwana it is very common in the kens or prefectures of Hokkaido, Aomori, Akita, Iwate, Yamagata and Nagano, all of which are in the northern and eastern portions of the kingdom.

BIOLOGY OF THE ERMINE MOTHS.

LIFE STAGES OF *padellus*.

Egg.— The eggs are deposited in masses, usually oval in shape, which are elongated in the general direction of the twig on which they are situated. The egg mass appears as a reticulated disk or pellicle, which is flattened but slightly convex, and is closely attached to the bark. The dimensions are generally from three to five millimeters in length and upwards of four millimeters in width, but the egg masses vary much in size as well as in shape, depending on the number of eggs they contain and their accommodation to the positions on the convex surfaces of the twigs. Not infrequently they very much resemble a eulecanium scale in its earlier stages of development. The individual egg has the appearance of a flattened, yellow, soft disk, oval in shape, with the central area slightly raised, and marked with longitudinal ribbings. It measures about seven hundred microns wide and eight hundred to nine hundred fifty microns in length. The eggs are arranged in rows and are superimposed on one another like tiles on a roof, the imbrication being very noticeable under slight magnification. The number of eggs in an assemblage is variable,[1] running in some instances to only a few, but ordinarily upwards of fifty to eighty in a mass. At the time of deposition the egg mass is covered with a glutinous substance which on exposure to the air forms a resistant protective covering. This is at first yellow, but with the progress of embryonic development it becomes mottled with red and later turns brownish or greyish brown, thus resembling in color the bark on which the eggs are attached. The eggs are usually placed near a bud of the current year's terminal growth and less frequently on the older wood.

First larval stage.— Length about 1 mm., ranging from about eight hundred to nine hundred and fifty microns; head, cervical

[1] The smallest number of *malinellus* eggs observed in a cluster on seedlings was nine, and the largest number was eighty-three, while the majority of egg masses had between thirty and forty eggs.

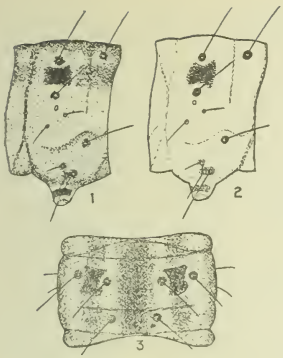
shield, anal plate and legs dark; body pale, but under magnification and with transmitted light it is dirty yellow in color and tuberculose; spiracles brownish, and those commencing with the fourth abdominal segment have above them a pigmented spot, bearing a spine; hairs on dorsum of thorax and abdomen fine and few in number. This description is based on caterpillars in the hibernating stage as taken from the egg mass.

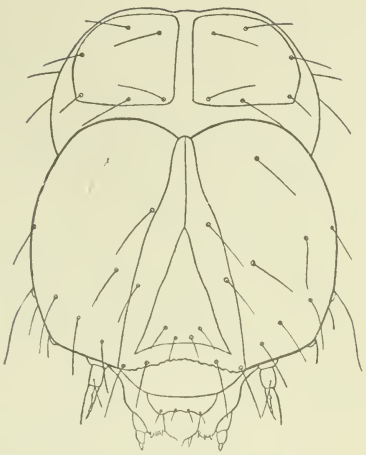
Mature larva.— The general characters of the larva are as follows: Head, thoracic shield and anal plate, black; anterior and posterior extremities much more narrow than remainder of body; head conspicuously notched behind; body pale, light yellow, dirty grey or greenish, sometimes appearing brownish on dorsum; four dark setigerous tubercles on dorsum of segments IV to XI inclusive; sides of segments with two rows of similar tubercles more widely separated; segments II to XI with two large dark, kidney-shaped pigmented areas subdorsally; average length 15 mm. Plate III.

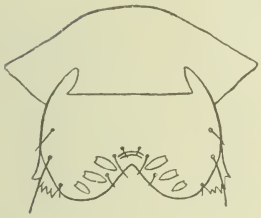
The following is a detailed description of the full-grown larva, or last instar: Length, 15 mm. average; greatest width 2 mm.; body cylindrical, attenuated at both extremities, especially on posterior part. The segments are outlined by constrictions, and are more or less distinctly divided into three annulets. Pile is very fine and visible only when magnified, otherwise the body appears naked except for a few fine, slender setæ that project from minute, almost obscure, tubercles; feet black and equal; prolegs rather short, with a semi-circular brown band on outer face of each foot; crochets three rows deep in a circle; anal plate black and of small size; anal-leg plate brown on dorsal and median portion and black on ventral side. Spiracles are small, slightly elliptical, that of segment XII being the largest.

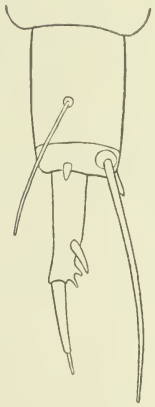
Head black, smooth, inclined to oblate in shape, conspicuously incised on posterior margin, sparsely covered with light-colored hairs; antennæ small; antennæ, epistoma and labrum brown; tips of mandibles dark or blackish brown; clypeus of medium height, acutely triangular, its lateral margins sinuate, and near its base four setæ arranged in an inverted arch; paraclypeal pieces long, narrowing toward bases, with two spines one above the other near apex of clypeus; ocelli six in number, with or without pigmentation, the group being protected by setæ.

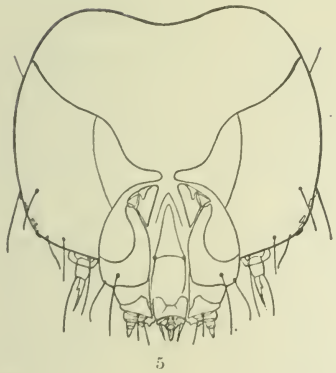
Cervical shield black, divided on median or deeply incised on posterior margin, with six fine, dark setæ arranged in rows of three on each lateral one-half of shield,— one row parallel to anterior margin and one row parallel to posterior margin; laterad of the shield, cephalad of spiracle, a small black pigmented area with three setæ of which the central one is longest; directly laterad two setæ from a small black pigmented area; spiracle oval and brownish.











LARVAL STRUCTURES.

1 and 3, Lateral and dorsal views of dark form; 2, light form, showing setæ and markings of abdominal segments; 4, setæ on front of head and thoracic shield of caterpillar; 5, rear of head, showing setæ; 6, antenna of caterpillar; 7, labrum of larva.

On segments II to XII a median dorsal and two sub-dorsal shaded areas which appear as three lines or bands. These lines vary in distinctness in different individuals. Each sub-dorsal band is interrupted by a distinct brown or black blotch on the second annulet of the IV to XI segments. The positions of the tubercles on segments V to IX are: — ii slightly dorsal to i, i being situated at the upper border of a sub-dorsal black patch; iii lateral; iv and v remote, iv being out of line with v, slightly below lower border of spiracle; vi posteriorly sub-vented; vii, of the three setæ on base of leg, the upper one not closely approximated to the other two; viii next midventral line. The arrangement of the setæ on segments IV, X, XI is similar to the above except that tubercle vii is represented by two setæ on segments IV and X, and by one seta on segment XI. On segment XII, i, ii, iii and viii are normal, and in the place of tubercles iv, v, vi and vii there are three or four setæ, occurring as pairs with some larvæ and in other specimens as one pair and a single seta. This difference in the number of these setæ was frequently noticed on the right and left sides of the same segment.

Pupa.— The head, wing covers and tip of abdomen usually blackish brown and the remainder of the body yellowish. This coloration seems to be quite constant for the insects reared on hawthorn, but pupæ from caterpillars reared on cherry were yellow or light orange, and dark about head and tip of abdomen. The cremaster consists of six spines, surrounding the anal end. The length is about 9 mm. and the width is 2.5 mm. Plate III.

Cocoon.— The cocoon is greyish and is from 10 to 12 mm. in length and 3 mm. in width. It is elongated oval in shape, though some specimens are distinctly pointed at one end, giving the cocoon the form of an oat or barley kernel. The cocoons from caterpillars reared on hawthorn are delicate and thin in texture so that the pupa within is generally visible. The cocoons from plum were more compact, and whitish. Plate IV.

Adult.— Head, palpi and antennæ white. Thorax white with a few black dots. Legs and abdomen white with a silvery sheen. Fore wings snowy white or greyish. The grey coloration is variable in extent and in depth of shade. Frequently the wings are entirely clouded or the grey marking appears as a blotch extending from the costa to the fold or merely as a streak along the outer or costal margin of the wing. The upper sides of the fore wings have usually from twenty to thirty or more dots principally distributed in three rows; one near costal margin and one on each side of the wing fold, with a variable number about the apex. Cilia pale grey or white with greyish tips. The undersides of the wings are grey or brownish grey and cilia grey. The hind wings are ashen-grey or fuscous, with fringe sometimes somewhat paler. Undersides grey, not differing appreciably from the upper surface. Expanse of wings 19 to 22 mm. Plate V.

LIFE STAGES OF *malinellus*.

Egg.— This is apparently not distinguishable from that of *padellus*. The egg masses are also of a similar appearance. Plate I.

Larva.— The mature caterpillar is apparently not different in its external characters from the *padellus* larva. The caterpillars reared on apple seedlings varied much in coloration, and the majority of them were yellow and lacked the median dorsal and sub-dorsal shaded areas which usually prevailed with the preceding species. Plates II and III.

Pupa.— Light orange yellow or brown, with the extremities sometimes dark. Length, 6–8 mm. Plate III.

Cocoon.— White and more densely woven than with preceding form. Cocoons are thickly massed in the tent. Plate IV.

Adult.— Similar to the light form of *padellus* and indistinguishable from it. Plates IV and V.

LIFE HISTORY AND HABITS.

Oviposition occurs principally during the latter part of June or during July, according to latitude and seasonal conditions, and hatching takes place in early autumn. The young larvæ at this season are inactive and remain sheltered through the winter under the protecting crust of the eggs. They abandon their winter quarters as the first leaves begin to unfold in the spring, making their exit through one of several tiny holes in the covering of the egg mass. The young caterpillars assemble among the tender leaflets of an adjacent bud and those of *malinellus* on apple bore into the parenchyma, beginning at the edge and usually near the apex of the leaf. As many as a dozen of the insects may exist as a colony within the pulpy substance of a single leaf. Within a few days after their entrance the leaves turn reddish at the points of injury, and those more severely mined may wither and die. Towards the end of the time of blossoming the caterpillars cease to burrow and feed openly on the leaves, concealing themselves with a greyish web. With the need of more food they extend their webs, seizing and involving fresh leaves in the common nest, on which they feed. Having destroyed the foliage on one branch they migrate " en masse " to another, and in severe attacks the trees may be defoliated and completely covered with a veiling, which becomes discolored by stains from the enclosed fragments of leaves and the dust-like excremental particles of the insects. On reaching maturity the larvæ spin their cocoons in contact with each other and, according to Mokshetsky, there may be during very destructive outbreaks as many as fifteen hundred cocoons placed side by side in regular rows within the silken tent. The moths make their appearance during the latter part of June or early July, and egg-laying commences

in about fifteen days after their emergence. The adults are motionless during the daytime, but with the approach of night they fly gently for short distances in a zig-zag course. The larvæ of *padellus* apparently do not have the mining habit; but, aside from the fact that they do not burrow into the leaves of their favorite host plants, as hawthorn, plum, etc., the life history and habits of this insect are similar to those of the above moth.

OCCURRENCE OF ERMINE MOTHS IN NEW YORK.

THE DISCOVERY.

The discovery of *padellus* in the State of New York was due to the close supervision of foreign importations of nursery stock during the spring of 1909 by the agents of the Division of Nursery Inspection of the New York Department of Agriculture. Special precautions were observed this year with such stock, which have since been followed, because many nests of the notorious "brown-tail moth" (*Euproctis chrysorrhœa* L.) were detected among the shipments, a source of danger from this insect which was not fully appreciated until this experience. After the setting out of the plants, the plantations were frequently examined for the appearance of destructive insects. Nothing was noted to arouse any suspicion until June 23 when Mr. John Maney, an official nursery inspector, detected three cherry seedlings completely covered with webs. The unfamiliar appearance of the nests and the enclosed caterpillars, coupled with the fact that the specimens were taken from foreign nursery stock, influenced him to bring the material to the Department of Entomology of this Station for identification. The plantation from which the insects were obtained was inspected again several times, and on June 24 five more infested cherry seedlings were secured. Examinations were made a little later in this and other similar plantings in different parts of the State to find more specimens of the caterpillars or traces of the insect but without success.

IDENTIFICATION OF SPECIES.

Nearly all the material collected during 1909 was immediately destroyed to avoid taking chances on the escape of any of the insects. Some caterpillars, however, were reared in the laboratory to obtain a few adults in order that the species should be correctly determined. Six moths were obtained, which were compared with descriptions by various authorities, and the insect was identified as *Yponomeuta padellus* L. A statement to that effect was published in a technical periodical.[1] To make certain the identity of the species which we

[1] *Jour. Econ. Ent.*, 2: 305. 1909.

had bred, several specimens of the moths were later sent to Dr. Paul Marchal of Paris, France, who has devoted considerable study to these insects and his report was a confirmation of our identification.

IMPORTATIONS FROM 1910 TO 1912.

During the spring of 1910 the local importations of foreign seedlings were very large. The same conditions prevailed with regard to the brown-tail moth, although the winter nests of this species were much less numerous than during the previous year. All stocks were carefully examined for evidences of other injurious insects and the usual precautionary measures were taken as before under the direction of the Division of Nursery Inspection. The local nurseries were carefully watched during June for the appearance of *Yponomeuta* caterpillars, and on the 24th of this month colonies of the insects were found on apple seedlings. Mr. G. G. Atwood, Chief of the Division of Nursery Inspection, was promptly notified of the discovery and instructions were at once sent to the inspectors in the field to make an immediate and careful canvass of all plantings of nursery stock set out during 1909 and 1910. During the following two weeks infestations were discovered in other plantings about Geneva and in nurseries about Orleans, Newark, Hilton, Schoharie, Blauvelt and Dansville; and from these, eight hundred and seventy-three colonies of caterpillars were obtained.

The plantings of foreign seedlings during 1911 were noticeably much more free of *Yponomeuta* nests than during the two preceding years. On June 8 a single nest of about fifty caterpillars was obtained at Lockport, and during the latter part of this month and early July a few infested plants were found about Geneva, Penfield, Chili, Johnstown and Schoharie. At the last-named locality empty cocoons of the insect were discovered which indicated that the caterpillars had pupated and the moths had made their emergence.

During 1912 the nests and caterpillars obtained were less in number than in any year since our attention has been called to the occurrence of these pests in this State. Infested seedlings were first detected at Seneca and later colonies of the insects were found in nursery plantings about Geneva and Rochester.

The following table showing the collections of colonies of the ermine moths in this State during these years is based on data which was kindly furnished by Mr. G. G. Atwood through the courtesy of Calvin J. Huson, Commissioner of Agriculture of the State of New York.

Map 1.—Localities in which Imported Seedlings have been Found, Infested with Ermine Moths.

TABLE 1.—ERMINE MOTHS COLLECTED IN NEW YORK DURING THE YEARS 1909–12.

Date.	Name of inspector.	Number of seedlings with nests	Kind of seedling.	Locality.
June 23, 1909	John A. Maney	3	Cherry	Geneva.
24, 1909	John A. Maney	5	Cherry	Geneva.
24, 1910	John A. Maney	6	Apple	Geneva.
25, 1910	John A. Maney	62	Apple	Geneva.
26, 1910	John A. Maney	110	Apple	Geneva.
27, 1910	J. J. Barden	10	Apple	Orleans.
28, 1910	James Goold	1	Apple	Schoharie.
28, 1910	P. L. Huested	8	Apple	Blauvelt.
28, 1910 to July 5, 1910	J. J. Barden	663	Apple	Orleans. Newark. Dansville.
June 29, 1910	James Goold	2	Apple	Schoharie.
30, 1910	John A. Maney	2	Apple	Geneva.
July 1, 1910	John A. Maney	8	Apple	Geneva.
5, 1910	J. A. Thompson	[1] 1	Apple	Hilton.
June 8, 1911	Bernard Blanch	[2] 1	Apple	Lockport.
19, 1911	J. A. Thompson	2	Apple	Penfield.
22, 1911	John A. Maney	5	Apple	Geneva.
28, 1911	J. A. Thompson	4	Apple	Chili.
July 6, 1911	James Goold	[3] 2	Apple	Schoharie.
7, 1911	James Goold	1	Cherry	Johnstown.
7, 1911	James Goold	2	Apple	Johnstown.
June 12, 1912	Bernard Blanch	[4] 1	Apple	Seneca.
19, 1912	Charles Darrow	[5] 3	Apple	Seneca.
25, 1912	J. A. Thompson	[6] 2	Apple	Rochester.
28, 1912	John A. Maney	[7] 1	Apple	Geneva.

[1] The nest at the time of delivery to the Station had five pupæ.
[2] This nest was reported as having about 50 caterpillars.
[3] On this date cocoons were formed and moths had escaped.
[4] This nest contained 3 caterpillars.
[5] Three, 14 and 36 caterpillars respectively.
[6] Nine and 16 caterpillars respectively.
[7] Five caterpillars.

NOTES ON IDENTITY OF APPLE SPECIES.

CHARACTERS FOR SEPARATION OF SPECIES.

The determination of the moth bred from cherry as *padellus* has already been mentioned, and it is now to be noted in Table I that by far the larger number of *Yponomeuta* larvæ were collected from apples. The question arises, "To which species do these belong,—to *padellus* or *malinellus?* The moths of the former, as has been previously indicated, are exceedingly variable in their markings, and unfortunately the identification of the two species seems to rest largely upon color distinction of the adult insects. In spite

of seeming morphological and biologic differences the separation of these insects is difficult and unsatisfactory, and there exists consequently considerable uncertainty as to the actual status of these two forms.

According to Dr. Marchal [1] " the ground color of the front wings is entirely white with *malinellus*, and more or less tinted with grey with *padellus*. The fringe of the anterior wings, examined from below, is whitish for the most part with *malinellus*, while with *padellus* it is grey or almost entirely grey. Finally the under-surfaces of the front wings of *padellus* are entirely grey, while the margins of the wings of *malinellus*, examined from below, are finely bordered with white. . . . . *padellus* is extremely variable, and the variation extends to the characters which are used to distinguish it from *malinellus*. Certain examples have the anterior wings largely tinted with grey, others have white wings, while some are intermediate. The fringes are also variable in their coloration, and the narrow white border of the lower surface is not always a constant character."

Some apparent differences in feeding habits and appearances of the caterpillars, and in the coloration of the pupæ and texture of the cocoons have also been noted. Lewis [2] in 1836 called attention to the fact that the larvæ of the *Yponomeuta* on apple upon emerging from the egg masses in the spring are leaf miners. This was verified by Delacour [3] in 1850 and Bissière [4] in 1876 and while this habit has been overlooked by many writers it has been described with much detail by Mokshetsky in his recent treatise. Marchal also records that *mahalebellus*,[5] a closely related species, similarly burrows into the leaves of the Mahaleb cherry. Curiously enough the mining instinct which manifests itself with the foregoing species has apparently not been observed or at least satisfactorily established for *padellus* caterpillars. Rebaté and Bernès also call attention to apparent preferences for host plants which, coupled with slight differences in the appearance of the moths, larvæ and pupæ are given by them to support the opinion that the insects represent distinct species. According to them branches of plums and apples may intercross, and, depending on which of the two forms is present one fruit will have the foliage eaten while the other will be immune. Moreover in some experiments conducted by them the larvæ of *padellus*, reared on plum, would not attack apple foliage; and vice versa caterpillars of *malinellus* taken from apple would not feed on plum. To the contrary Gruvel [6] states that in a test conducted

[1] *Bul. Soc. d'Étud. et Vulg. Zool. Agric.* p. 23, 1902.
[2] *Trans. Ent. Soc. London* 1:21–22, 1836.
[3] Essai sur les Insectes, 1850, p. 296.
[4] *Bul. d' Insectologie Agricole*, No. 4, p. 83, 1876.
[5] *Bul. Soc. d'Étud. Vulg. Zool. Agric.*, p. 21, 1902.
[6] Quoted from La Chenille Fileuse, Rebaté and Bernès.

by him young larvæ of *malinellus* taken from apple subsisted equally well on plum foliage and mined the leaves, as is their normal habit on their favorite host. The opinion of Schöyen that *padellus* is the common species on apples in Norway and the observation by Bos of *padellus* migrating from Cratægus to apple trees have already been mentioned.

The differences noted by Rebaté in the appearances of the larvæ and cocoons of *padellus* as reared on plums in comparison with these stages of *malinellus* bred on apple are as follows: — The caterpillar of the former species is of a yellowish-grey color. The cocoons are thin in texture, of a greyish-white color and more or less detached from one another in the tent, while the caterpillars of the latter species are lighter in color and a little shorter and more slender. The cocoons are white, more dense and compact, and are attached one to another forming clusters or packets which vary in size according to the numbers of the caterpillars in the colony. Theobald observes similar differences in the cocoons and adds that the nest or tent is likewise not nearly as compact with *padellus* as with the apple-feeding species.

COMPARISONS OF COLLECTIONS OF *padellus* AND *malinellus*.

Bearing in mind the foregoing distinctions it is now of interest to compare collections of these insects from various geographical areas to note the range of variation and the forms that are in the region of their occurrence understood as constituting *padellus* and *malinellus* respectively. There is also included in this comparison the material bred from apple and cherry in New York.

Padellus.	*Malinellus.*
Habitat,[1] Germany.	Habitat,[2] Germany.
Host plant, hawthorn.	Host plant, apple.
Moth, primaries, including fringe, clouded and dark. Expanse of wings 22 to 23 mm.	Moth, primaries entirely white or white with exception of fringe which may be slightly clouded. Expanse of wings 19 to 20 mm.
Larva, dark olive-green with dorsal spots rather indistinct. Length 15 to 17 mm.	Larva, pale, dirty white or greenish-yellow with dorsal spots distinct. Length 10 to 12 mm.
Pupa, head, wing pads and tip of abdomen dark brown or black and remainder of body yellow. Length 7 to 9 mm.	Pupa, pale or brown. Length 6 to 8 mm.
Cocoon, greyish-white, thin and pupa visible.	Cocoon, white, densely woven, and concealing pupa.
Habitat,[3] France.	Habitat,[4] France.
Host plant, hawthorn.	Host plant, apple.

[1] Collection, 2 adults, 3 larvæ and 12 pupæ from Dr. L. Reh, Hamburg, Germany
[2] Collection, 7 adults, 3 larvæ and 4 pupæ from Dr. L. Reh, Hamburg, Germany.
[3] Collection, 2 adults, 4 larvæ and 3 pupæ from M. J. de Joannis, Paris, France.
[4] Collection, 17 adults, 3 larvæ and 5 pupæ from M. J. de Joannis, Paris, France.

Padellus.

Moth, primaries clouded, with apex and fringe noticeably darker. Expanse of wings 17 to 18 mm.

Larva, dirty-white, greenish-yellow, or olive green, dorsal spots distinct with light forms. Length 12 to 16 mm.

Pupa, head, wing pads and tip of abdomen dark brown or black; remainder of body yellow. Length 8 to 9 mm.

Habitat,[2] Scotland.
Host plant, apple.
Moth, primaries white, expanse of wings 16 to 20 mm.
Larva, pale with conspicuous dorsal spots or dark olive-green with markings less distinct. Length 10 to 12 mm.
Pupa, pale to yellowish with extremities brownish. Length 6 to 8 mm.
Cocoon, white and of dense texture concealing enclosed pupa, in compact clusters.

Habitat,[3] Holland.
Host plant, hawthorn.
Moth, primaries generally dark showing cloudy or lead-colored areas about fringe or costal margin. Few specimens almost white. Compared with moths reared on apple this collection appears on the whole quite dark. Expanse of wings 21 to 22 mm.
Larva, pale to dark olive green. Length 15 to 17 mm.
Pupa, head, wing pads and tip of abdomen black; remainder of body yellow. Length 7 to 10 mm.
Cocoon, greyish white and of thin texture showing the pupa.

Malinellus.

Moth, primaries usually entirely white, but quite a few specimens have fringe slightly shaded. Two specimens have a very distinct grey fringe and a dark blotch along costal margin. Expanse of wings 16 to 22 mm.
Larva, pale, and dorsal spots distinct or dark olive in color with dorsal markings less conspicuous. Length 11 to 15 mm.
Pupa, light orange yellow with head and tip of abdomen dark brown or blackish. Length 7 to 10 mm.

Habitat,[1] Hungary.
Host plant, apple.
Moth, primaries white with only an occasional specimen with clouded apex or fringe. Expanse of wings 18 to 20 mm.
Larva, pale and dorsal spots prominent. Length 15 to 16 mm.
Cocoon, white, dense and thickly massed.

[1] Collection, 31 adults, 3 larvæ and 10 cocoons from F. A. Cerva, Szigetcsep, Hungary.
[2] Collection, 7 adults, 10 larvæ and 3 pupæ from Prof. R. Stewart MacDougall, Edinburgh, Scotland.
[3] Collection, 20 adults, 3 larvæ and 8 pupæ from Prof. J. Ritzema Bos, Wageningen, Holland.

Padellus.	*Malinellus.*
	Habitat,[1] Japan.
	Host plant, apple.
	Moth, primaries including fringe usually white, but fringe is sometimes slightly clouded. One moth also shows shading on costal margin. Expanse of wings 20 to 22 mm.
	Larva, dark olive green. Length 12 to 15 mm.
	Pupa, head, wing pads and tip of abdomen dark brown, and about constrictions of abdominal segments lighter brown. Length 10 to 12 mm.
	Cocoon somewhat thin in texture, greyish or white in color, and thickly massed.
Habitat,[2] New York.	Habitat,[3] New York.
Host plant, Mahaleb cherry.	Host plant, crab.
Moth, primaries white with fringe slightly clouded. Expanse of wings 18 to 19 mm.	Moth, primaries white or white with fringe slightly clouded. Expanse of wings 18 to 20 mm.
Larva, pale or greenish-yellow or olive green. Length 12 to 16 mm.	Larva, pale, greyish-brown or dark greenish yellow. Length 12 to 15 mm.
Pupa, light orange yellow and dark brown or black about head and tip of abdomen. Length 8 to 9 mm.	Pupa, light orange yellow and some specimens with extremities dark. Length 6 to 8 mm.
Cocoon, white and concealing pupa.	Cocoon, usually white and densely woven, but some specimens were thin in texture, showing the pupæ.

From the foregoing comparisons it will be observed that the adults of *padellus* from hawthorn and of *malinellus* from apple represent for the most part extremes in wing coloration. The former contains a majority of moths which have the primaries and fringes clouded, greyish or lead colored, while the latter has a majority of moths with primaries and fringes white. The two are distinct enough when characteristic examples are selected, but the separation of them becomes difficult when the intergrading forms are considered, as they merge into each other by imperceptible gradations.

The larvæ of both forms are quite variable in color but they present no structural differences. In the collections from apple seedlings pale forms predominated. The pupæ and cocoons of the insects from hawthorn as shown by Rebaté consistently differ from those taken from apple, but no constant differences were observed in the material collected from cherry in comparison with that obtained from apple. If any differences exist in the insects reared by us from cherry and apple they are principally that the moths

[1] Collection, 14 adults, 15 larvæ and 3 pupæ from Dr. S. I. Kuwana, Tokio, Japan.

[2] Collection, 6 adults, 12 larvæ and 2 pupæ from imported seedling cherries growing in nursery plantations about Geneva.

[3] Collection, 76 adults, 16 larvæ, 7 pupæ from imported apple seedlings growing in nursery plantations about Geneva.

reared from cherry invariably had shaded areas along the outer margins of the wings, while those from apple had for the most part white primaries.

Taking all characters into consideration, the prevailing white anterior wings of the adults, habits of larvæ, coloration of pupæ, texture and massing of cocoons in the web, the species we have reared on apple seems unquestionably to be identical with the form commonly known in Europe as *malinellus*.

CROSS-FEEDING EXPERIMENTS.

The contradictory results obtained by various writers in feeding tests with *padellus* and *malinellus* by interchanging their host plants led us to make some tests along similar lines. Three small sections of wood, each containing a colony of *padellus* larvæ, were, at about the period for their migration from the egg masses, placed near opening apple buds and slightly moistened each day to prevent drying. None of the larvæ emerged and all eventually died. Later living larvæ were taken from their hibernating quarters on cherry and transferred to apple buds which were just showing the tips of the first leaves. These apparently did no feeding, nor did they make any efforts to burrow into the apple leaves. After struggling for several days on the surfaces of the leaves and frequently precipitating themselves to the ground, they finally succumbed. Their efforts were feeble as if they had suffered from the handling and confinement during storage of the nursery stock or the conditions incidental to their removal or opening of their hibernating quarters were abnormal and injurious to them.

Tests with older caterpillars of *malinellus* were more satisfactory. Twenty full-grown specimens of *malinellus*, reared on imported apple seedlings, were placed in a cage containing twigs from Baldwin apple and Montmorency cherry. Webs were at once spun over both fruits but the insects fed only on the apple and apparently made no effort to attack the cherry. This experiment was repeated with a similar number of insects, but only a single twig of apple was used which was placed in the center of a number of shoots of Montmorency cherry. The caterpillars quickly selected the apple twig and after consuming the apple leaves they extended their webs over the cherry foliage but in no case did they feed upon it.

A third test was then made with thirty caterpillars which were confined to a young Mahaleb cherry seedling. They were at first very restless and seemed to exhibit an aversion for the foliage; but later this was overcome and apparently under the stress of hunger several were observed to eat the leaves with relish. Most of the insects, however, fed very little. Twigs of an imported apple seedling were then introduced into the cage which were attacked in a ravenous manner. In spite of an abundance of apple foliage a caterpillar was occasionally observed nibbling on cherry leaves.

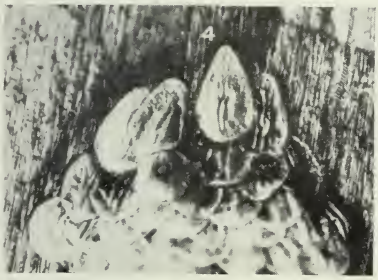


PLATE I. — LIFE STAGES OF *Yponomeuta malinellus*.
(See reverse of Plate IX.)

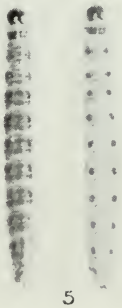
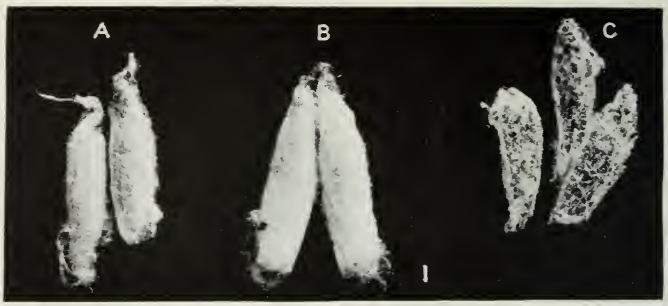


PLATE II.— CATERPILLARS OF *Yponomeuta malinellus*.
(See reverse of Plate IX.)

PLATE III — LIFE STAGES OF *Yponomeuta padellus* AND *malinellus*.
(See reverse of Plate IX.)



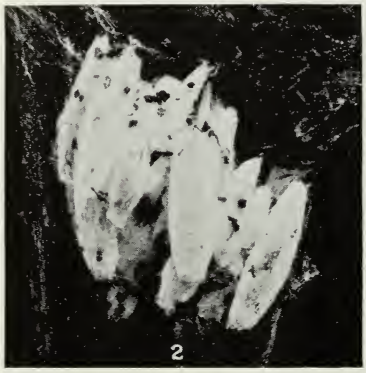
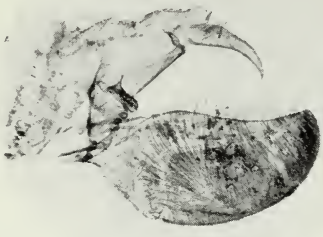


PLATE IV.— LIFE STAGES OF *Yponomeuta padellus* AND *malinellus*.
(See reverse of Plate IX.)

PLATE V.— SOME YPONOMEUTA MOTHS.
(See reverse of Plate IX.)



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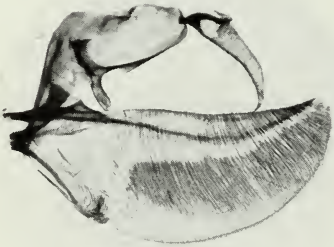

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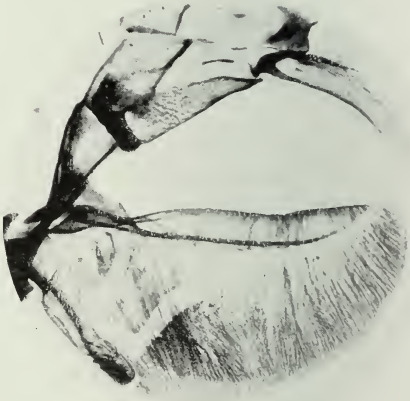

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PLATE VI. — STUDIES ON GENITALIA OF YPONOMEUTA MOTHS.

(See reverse of Plate IX.)

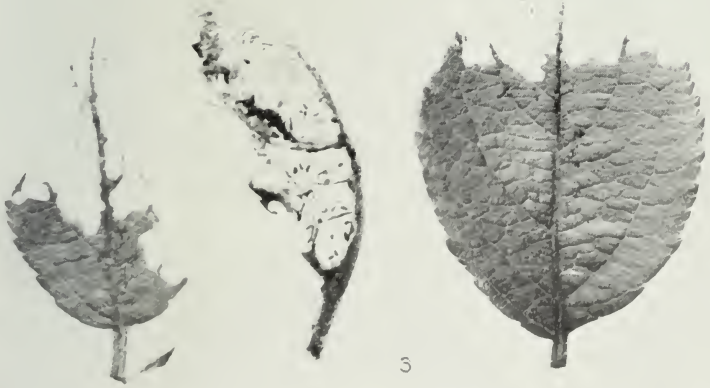


PLATE VII.— FEEDING HABITS OF *Yponomeuta malinellus* ON APPLE.

(See reverse of Plate IX.)

PLATE VIII. — APPLE SEEDLING SHOWING DEFOLIATION AND WEBS OF *Yponomeuta malinellus*.

PLATE IX.— CHERRY SEEDLING SHOWING WEBS OF *Yponomeuta padellus*.

EXPLANATION OF PLATES.

PLATE I.— LIFE STAGES OF *Yponomeuta malinellus*.

1, 2, 3, and 4, egg clusters, natural size and enlarged; 5, egg mass reversed showing hibernating larvæ; 6, larvæ enlarged; 7, apple leaf showing "mined" areas.

PLATE II.— CATERPILLARS OF *Yponomeuta malinellus*.

Characteristic positions on leaves and in webs, and range of variability in color and markings.

PLATE III.— LIFE STAGES OF *Yponomeuta padellus* AND *malinellus*.

1, Pupæ of *padellus*, and 2, of *malinellus*; 3 and 4, dorsal views of dark and light-colored caterpillars; 5, caterpillar, lateral view showing characteristic markings.

PLATE IV.— LIFE STAGES OF *Yponomeuta padellus* AND *malinellus*.

1 a and c, cocoons of *padellus* on cherry and hawthorn; 1 b, cocoons of *malinellus* on apple; 2 and 3, cocoons of *malinellus* (enlarged) and position on infested seedling; 4, *malinellus* moths in resting positions; 5, moth enlarged.

PLATE V.— SOME YPONOMEUTA MOTHS.

1, *Yponomeuta padellus* L.; 2, intergrading form of *padellus* and *malinellus*; 3, *Y. malinellus* ZELL.; 4, *Y. evonymellus* L.; 5, *Y. multipunctellus* CLEM.; 6, *Y. mahalebellus* GN.

(Enlarged.)

PLATE VI.— STUDIES ON GENITALIA OF YPONOMEUTA MOTHS.

1, *Yponomeuta padellus* from hawthorn; 2, *Y. padellus* (*malinellus*) from apple, Scotland; 3, *Y. padellus* from cherry, Geneva, N. Y.; 4, *Y. malinellus* from apple, Japan; and 5, *Y. malinellus*, apple, Geneva; 6, *Y. polystica* (Sent by Kuwana).

PLATE VII.— FEEDING HABITS OF *Yponomeuta malinellus* ON APPLE.

1, Spinning of web preparatory to feeding; 2, foliage consumed; 3, character of feeding on apple leaves.

PLATE VIII.— APPLE SEEDLINGS SHOWING DEFOLIATION AND WEBS OF *Yponomeuta malinellus*.

PLATE IX.— CHERRY SEEDLING SHOWING WEBS OF *Yponomeuta padellus*.

COMPARISONS OF GENITALIA.

With these moths the uncus is long and slender, and it is sinuate towards the tip which is acute and a little hooked. The lateral pieces or claspers are broad and hemispherical, with the apices gently rounded or somewhat acutely rounded according to their positions in the mounts. The sexual parts of the male are symmetrical or very closely so, and the general type of the genitalia of this group of moths is shown in Plate VI.

A study of a goodly series of mounts of *padellus* from hawthorn and *malinellus* from apple from different geographical areas reveal no tangible structural differences between them. The lobe or spur at the base of the uncus varies slightly in width and length with some individuals, but this variation may also be detected with any assemblage of males from either of these hosts. A comparison of the figures, Plate VI, plainly shows that there are no obvious distinctions in the more important organs which will bear out these minor differences.

Considering all differential features, structural as well as superficial, it appears that the moths of *malinellus* do not possess sufficiently diverse values to entitle them to specific distinction and that the specimens bred from hawthorn, cherry and apple really constitute a single species. Breeding experiments are now needed to definitely settle the status of these two moths, and these we have not undertaken as it did not seem wise to take chances with the insects. We have, therefore, followed the example of European writers, and have treated the two forms as distinct species.

THE ERMINE MOTHS ON SEEDLINGS.

ORIGIN OF INFESTATION.

The stock which has been responsible for the introduction of the ermine moths consists of one-year-old seedlings, which are frequently grown in plats near hedge rows, trees or even woodland. Such surroundings are very favorable for the breeding of various destructive pests. The flight of the ermine moths takes place during July and August, and in their excursions some of them have unquestionably made their way to the nursery blocks in the immediate vicinity and deposited their eggs on the young seedlings, which were subsequently shipped to the United States. The life history of the insect would indicate this course of events, and since we have become familiar with their appearance we have collected the egg masses on the stocks after their shipment to this country. The conditions during 1909 with respect to these pests surrounding some of the foreign nurseries which have been growing seedlings

for purposes of exportation are indicated by Dr. L. O. Howard in the following communication:[1]

"I saw them (*Yponomeuta* spp.) everywhere on my recent trip in France, and especially upon the hedges and trees at the borders of the plats of seedlings being grown for exportation to America. I saw *Yponomeuta* larvæ in their webs on almost every apple tree, sometimes only here and there a twig with some leaves webbed together, and occasionally considerable numbers of these webs." During years favorable for the multiplication of these insects, the chance of nursery stock becoming infested when grown under such circumstances is obviously very great, as has been well demonstrated.

FEEDING HABITS OF THE CATERPILLAR.

Before transplanting in the nurseries the seedlings are stubbed, making a plant which, including the root, measures from fifteen to eighteen inches in length, while the stalk has a diameter of about one-quarter of an inch and bears from five to ten leaf buds. The egg masses of the insects are generally found on the stalk within six to nine inches of the ground, and from one to three egg masses have been detected on a plant. These were placed just under or above a leaf bud, almost touching it, or in positions intermediate between two buds. Opportunity has not been afforded to observe the early movements of the larvæ but, judging from the conditions of leaf clusters, it would appear that the young caterpillars on emerging from the egg mass preferred the nearest opened bud above them, while dormant buds were passed by unharmed. Our observations indicated that the caterpillars on apple seedlings were, at this stage, leaf miners. The first leaves attacked by them showed along the margins near the tips reddish or rusty-colored blotches of varying sizes, and as a result of this injury the leaves were small as if stunted, while others were one-half destroyed or entirely killed. On abandoning their "mines" the caterpillars ascended higher on the seedlings and, on June 12, when first detected, were feeding openly on some of the upper leaf clusters on the central stalk of the plants or at the base of the terminal offshoots which were then from three to four inches in length. They spun a filmy tangle of fine silken threads between two leaves and proceeded to consume the upper pulpy tissues of the under leaf, while the lower epidermis was seldom or little eaten and served as the floor of their feeding grounds. The rejected portion of the leaf was at this time of a thin papery nature, light brown or reddish-brown in color, showing plainly the network of little veinlets, and is characteristic of the work of the insects at this stage. The caterpillars are social insects, feeding

[1] Letter of Aug. 18, 1909.

together on a single leaf, and as one leaf is consumed a fresh one is involved in the web, to be destroyed in like manner. By these operations there was soon formed a nest which, on being dissected, was found to be composed of the remnants of several leaves tightly woven together by silken threads. Interspersed in the cluster were the caterpillars themselves together with their molted skins and excremental particles. The nests were subsequently abandoned and the caterpillars marched "en masse" to higher leaf clusters or to the top of a terminal shoot, spinning webs as they advanced. As they approached maturity during the latter part of June the caterpillars were more active and ravenous. Whole leaf clusters were covered with webs and then completely devoured with the exception of the midribs, larger veins and stems. The injuries to the seedlings varied somewhat in extent but colonies of caterpillars from one dozen to two dozen in number usually completely defoliated a plant, while in the axils of the shoots and stretching from the tips of each shoot to the central stalks of the seedlings were the tenuous webs of the insects. Plates VIII and IX. The tents were at first whitish and compact, but on exposure to the weather and from stains due to moisture acting on the excrement and fragments of leaves they became discolored and ragged. On pupating, towards the latter part of June, the caterpillars spun their cocoons in the webbing in the tops of the seedlings; and in this operation, as in feeding, the social instinct was strongly manifested. As if by a given signal the larger number of them ceased feeding and, abandoning the foliage and taking positions in the web that were parallel to and apparently of equal distance from their neighbors, they spun their cocoons side by side, forming a cluster as illustrated in Plate IV.

BREEDING RECORDS.

Some caterpillars of *padellus* were observed feeding on cherry seedlings on June 23, 1909, and judging from their sizes and markings, all of them were at this time apparently mature. On June 28 some of the larvæ commenced to pupate, and on July 9 the first moths made their appearance. At this latter date a few caterpillars had not yet spun up. The moths lived in the breeding cages through July and one specimen survived until August 18.

In 1910 mature caterpillars of *malinellus* were found on apple seedlings on June 21. Five days later they commenced to spin cocoons and nearly all of them were in the pupal state by the first week in July. A few moths appeared July 6 but the adults were out in their largest numbers about July 14. Although the majority of them had transformed a few larvæ were still unchanged at this latter date. Egg deposition was first observed on July 18, when practically all of the moths had emerged. One moth lived until August 10.

In 1911 some caterpillars continued to feed on apple leaves until June 30. Pupation commenced June 26 and continued until July 6. Adults first appeared on July 10, and some continued to emerge until July 17. One moth lived until August 15.

During 1912 a colony of caterpillars, apparently in the second larval instar, was collected on June 12 and these on June 24 were in the fourth instar. On July 1 the caterpillars began to pupate and the last cocoon was spun July 17. Moths made their appearance on July 10 and some continued to emerge until July 15. One moth which was confined in a breeding cage lived until August 17.

FUTURE IMPORTANCE OF THE INSECTS.

The occurrence of the Yponomeuta caterpillars in New York during recent years raises the question as to the rôle these insects are destined to play as fruit pests in the United States. This cannot be answered satisfactorily as so little data is available upon the actual behavior of these lepidopterons in this country. Our knowledge regarding them in New York is limited solely to a small territory about nursery plantings in certain nursery centers, and if they exist in other states they have apparently not attracted attention. With the ability of these insects to survive the conditions incidental to the importation of nursery stock from abroad and to escape the ordinary nursery inspection, the wonder is that they have not before this succeeded in establishing themselves along the avenues of trade in America. For it is to be noted that in New York a close supervision over shipments and plantings of imported nursery stock has only been maintained since 1909 and the condition of foreign purchases with respect to the ermine moths and other dangerous species as a result of more rigid inspection is well known. If earlier importations were as commonly infested with these pests as they have been during the past four years it would seem not improbable that somewhere these moths have made their escape from nurseries to adjoining plantings where perhaps they have secured a foothold. In states where there has been no such inspection the danger that such has taken place is obviously much greater.

Since the discovery of the ermine moths in this State the Division of Nursery Inspection has taken special precautions with imported stock and whenever infested plants have been detected they have been destroyed. In addition the surroundings of nurseries have also been inspected and there has so far been no evidence that these lepidopterons have gained a footing in New York. Nevertheless pests of foreign origin have entailed such great losses upon our farmers that it would be unwise for the nursery-inspection service in all of the states not to recognize the danger threatened by these moths and seek by precautionary and other measures to prevent them from becoming permanently established in this country.

A NATIVE SPECIES OF ERMINE MOTH.

There is one native species of the genus Yponomeuta which is *multipunctellus* Clem. Dyar records the Atlantic states as its range of distribution; and according to Chambers it is very common in Kentucky, while Gaumer has obtained specimens in Kansas. The caterpillar feeds on the leaves of *Evonymus atropurpureus* Jacq. and spins its webs over the plant as is characteristic of the insects of this genus. This species differs from European forms by the larger number of black dots on the front wings and the marked difference in the hind wings of the sexes. All the wings of the male are white while the female has the anterior wings white and the posterior wings dark gray.

NATURAL ENEMIES.

The ermine moths have a large number of natural enemies, the most important of which belong to the orders Hymenoptera and Diptera. Ratzeburg [1] has enumerated over thirty hymenopterous species which are said to attack these insects. In southern Russia, according to Mokshetsky [2] twelve species of hymenopterons and three species of dipterons prey upon *malinellus* and during some seasons they exert a marked regulatory and repressive action upon the multiplication of this pest. A common and most efficient enemy of both *padellus* and *malinellus* is the remarkable chalcid, *Encyrtus* (*Ageniaspis*) *fuscicollis* Dalm., which presents the exceptional phenomenon of polyembryony [3] and possesses immense reproductive powers.

Among collections of Yponomeuta moths received from Europe there were included a number of unnamed parasites which, through the courtesy of Dr. L. O. Howard have been identified [4] as follows: *Herpestomus* n. sp., *Angitia* sp. and *Tetrastichus* sp. from *malinellus* from France; *Discochæta evonymellæ* Ratz. from *padellus* from France; *Cnemedon vitripennis* Meigen from *padellus* from Holland; and from *malinellus* from Japan, *Herpestomus* n. sp. which according to Kuwana is the only parasite which attacks this lepidopteron in this country.

In spite of the large number of Yponomeuta caterpillars which have been found in New York, it is worthy of record that we have not reared any of the well known parasites which abound in the normal range of distribution of these pests. The failure of the more common and efficient species to accompany the ermine moths

[1] Die Ichneumon. d Forstins. Bd. 3, p. 259.

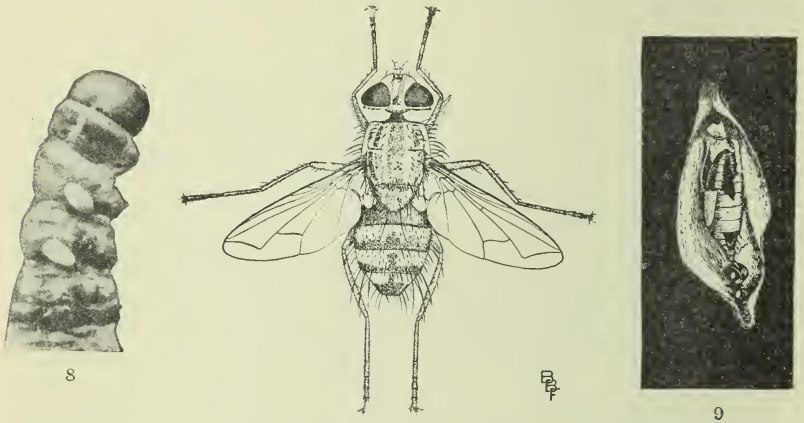
[2] The Apple Moth, 1907.

[3] Marchal, Paul. Recherches sur la Biologie et le Developpement des Hyménoptères Parasites — La Polyembryonie Spécifique ou Germinogonie, *Arch. Zool. Exp.*, 4, 2:257–335, 1904.

[4] Identifications of Hymenoptera by Mr. J. C. Crawford, and of Diptera by Mr. J. R. Malloch.

is a striking illustration of how foreign insects upon their introduction into the United States may find their struggle for existence a comparatively easy one, and by virtue of the balance in their favor become a serious item for economic consideration.

From *padellus* taken from cherry seedlings in New York we have bred a few specimens of *Mesochorus* sp., while the most common parasite of both *padellus* and *malinellus* was the tachinid, *Exorista arvicola* Meig. Some colonies had as many as 25 per ct. of the caterpillars carrying from one to three eggs of this fly, which were in the constrictions principally of the head and thoracic segments. The eggs are of a cream color and measure about .52 mm. long,



8 10 9

An Ermine Moth Parasite, *Exorista arvicola* Meigen.
8, Eggs on *malinellus* caterpillar; 9, puparium in *malinellus* pupa; 10, adult.
(All figures enlarged, last greatly)

.33 mm. wide and .19 mm. high. They are oval in shape, one end being broader than the other and are convex on the upper side. The surface is smooth and is covered with a delicate tracing of raised lines which give the appearance of a network of cells, pentagonal or hexagonal in outline. In hatching, a crack forms around the base about the wider end and extends upwards around the sides to about the middle. The portion above the crack raises up like a lid. The eggs of *arvicola* were first observed on mature caterpillars on June 25, 1912, which began to pupate on July 2. Moths from non-parasitized caterpillars commenced to emerge on July 10, while the tachinids appeared from July 10 to July 12.

A capsid,[1] *Atractotomus mali* Meig., is listed as an enemy of the ermine moths, and starlings [2] are said to feed upon the caterpillars.

[1] Pommerol, *Rev. sc. Bourbonn.* 14:18–23, 1901.
[2] Theobald, 2nd Rept. p. 35, 1904.

METHODS OF CONTROL.

The present situation regarding the ermine moths suggests the great importance of a careful inspection of nurseries, especially of the plantings of foreign-grown seedlings. Owing to the inconspicuousness of the egg masses, due to their small size and their color, which resembles that of the bark, very few of them are likely to be detected at the customary examination at the time of spring deliveries when the stock is being unpacked and sorted. The most effective work can be done during June, when the inspector should look for plants which show the webs or tents of the insect. Plates VII and VIII. All infested plants should be uprooted and destroyed. Experience has demonstrated very clearly the importance of more than one examination, and if two are made one inspection should be planned for the latter part of June when the work of the insects will be more conspicuous because the caterpillars are then full grown and will have spun their larger webs. If the work is delayed beyond this time there is danger that the insects may have pupated and transformed to moths. As insects may have escaped from previous infestations, premises adjoining nurseries should be similarly examined.

The caterpillars are quite susceptible to arsenical poisons and should it ever become necessary to combat them in plantings of older trees little or no modification will probably be required in existing spraying practices for orchards.

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[1] Titles marked by asterisk (*) have not been verified.

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THE ORGANIC PHOSPHORIC ACID OF COTTONSEED MEAL

R. J. ANDERSON

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TECHNICAL BULLETIN No. 25.

THE ORGANIC PHOSPHORIC ACID OF COTTONSEED MEAL.

R. J. ANDERSON.

SUMMARY.

Cottonseed meal contains an organic phosphoric acid which is very similar to phytic acid. When heated in a sealed tube with dilute sulphuric acid it decomposes into inosite and phosphoric acid. Whether the substance is identical with phytin could not be determined. The acid gives easily crystallizing barium salts.

The aqueous solution of the free acid gives all those reactions which have been attributed previously to the presence of pyro- and metaphosphoric acids in cottonseed meal.

The acid when given in 0.5 and 1 gram doses to rabbits does not show any marked toxic properties. Symptoms of distress were produced but the animals recovered their normal appearance after two or three hours.

INTRODUCTION.

In the investigation of the organic phosphoric acids present in various cereals and feeding stuffs which is being carried out in this laboratory, cottonseed meal was also examined. Earlier work by other investigators has shown that this product probably contains some complex organic phosphoric acid.[1] It seems, however, that if such a substance is present it has not been isolated in pure form nor have its properties been fully studied.

The opinion seems to be generally held that cottonseed meal contains some poisonous principle, but the exact nature of this principle has never been definitely determined. It has been claimed that pyro- and metaphosphoric acids were present in cottonseed meal[2] and it was thought that the poisonous properties of the product were due to the presence of salts of these acids. More recent work by Crawford[3] led him to believe that the poisonous principle was a salt of either a simple inorganic or a complex organic pyrophosphoric acid.

The presence of these acids has been adduced from the fact that the extracts of cottonseed meal give reactions similar to those of the above acids, viz., anomalous behavior towards ammonium molybdate, white precipitates with silver nitrate and coagulation of egg albumen; further, the poisonous effects resemble those given by these acids. Aside from these reactions, however, there is no

[1] Rather, Texas Agr. Exp. Sta. Bull. 146 (1912).
[2] Hardin, So. Carolina Agr. Exp. Sta. Bull. 8, New Series (1892).
[3] Crawford, *Jour. Pharm. and Exper. Ther.* 1: 519 (1910).

proof whatever that either pyro- or metaphosphoric acid is present in cottonseed meal.

The purpose of the present investigation was to isolate and identify, if possible, the organic phosphoric acid in cottonseed meal. We are, consequently, unable either to deny or affirm the absence or presence of pyro- or metaphosphoric acid in this product. We have found, however, that the organic phosphoric acid isolated from cottonseed meal gives all the reactions reported by the above authors, which they considered as evidence of the presence of pyro- and metaphosphoric acid. It seems, therefore, probable that the reactions referred to are due to the organic phosphoric acid rather than to pyro- or metaphosphoric acids.

The preparation of the substance and its purification will be more fully described in the experimental part. It will suffice to state here that cottonseed meal was extracted with 0.2 per ct. hydrochloric acid and the substance isolated as the barium salt. The purification of the substance is very difficult. The extract contains large quantities of soluble impurities, mucilaginous substances, proteins, etc., which render the purification extremely difficult and tedious. In addition to the above, there is apparently some carbohydrate associated with the organic phosphoric acid, the removal of which requires much time. For the same reasons the yield of the pure product is very unsatisfactory.

The compound finally obtained is very similar to phytic acid so far as composition and reactions are concerned. In fact it is impossible, from the present data, to determine whether the substance is phytic acid or an isomer. Both yield inosite when heated in a sealed tube with dilute sulphuric acid and the reactions of aqueous solutions of the free acids can hardly be differentiated.

The most striking difference is that the barium salt of the product from cottonseed meal shows a decided tendency to crystallize, a property which we have never observed when working with barium phytate under the same conditions.

If the substance from cottonseed meal is precipitated from acid solutions with barium hydroxide it separates as a white amorphous precipitate. When the dried precipitate is digested in 0.5 per ct. hydrochloric acid it dissolves very readily but after a few minutes it precipitates again. Under the microscope this precipitate is seen to consist of balls or globular masses of very fine needle-shaped crystals. The dilute hydrochloric acid solution of the barium salt gives a white amorphous precipitate on the addition of alcohol; by standing for several hours, however, it slowly assumes the same crystalline form as mentioned above. The free acid is not precipitated by barium chloride but if such a solution is allowed to stand over night or longer the barium salt will separate in fine needle-shaped crystals, grouped in the same general form as above but the individual crystals are much larger. The amorphous precipitates

are very soluble in 0.5 per ct. hydrochloric acid but after the substance has assumed the crystalline form, it is very slightly soluble in this medium.

While the barium salt was easily obtained in crystalline form it did not contain a constant amount of the base.

The variations would sometimes amount to as much as 3 or 4 per ct., depending upon the amount of the base present in the solution and the conditions under which the substance separated. In the presence of a large excess of barium chloride a salt corresponding nearly to tetrabarium phytate crystallizes out; when a small amount of barium chloride is present salts showing the above mentioned variations are formed; but when the substance has been repeatedly separated from acid solutions with alcohol a salt is obtained which corresponds nearly to tribarium phytate.

The aqueous solution of the free acid gives a heavy white amorphous precipitate with excess of silver nitrate; with ammonium molybdate a heavy white crystalline precipitate is produced which remains unchanged in the cold for a long time but when heated it soon turns yellowish in color. These reactions are identical with those given by phytic acid; with other metals both acids give apparently identical reactions.

The dilute aqueous solution of the acid coagulates egg albumen at once. This property, however, is not peculiar to the acid from cottonseed meal. Phytic acid was found to produce an identical effect. The tetraphosphoric acid ester of inosite[1] and the pyrophosphoric acid esters of inosite[2] mentioned in former papers also gave the same reaction. In view of the fact that the last mentioned substances coagulate egg albumen, it appears probable that this property is common to organically bound phosphoric acids.

As will be noticed from the foregoing the organic phosphoric acid of cottonseed meal gives all the reactions previously attributed to the presence of pyro- and metaphosphoric acids. But the question whether or not it is also the toxic principle in cottonseed meal remains unanswered. Preliminary experiments carried out with the acid obtained from the purified barium salt on rabbits are not conclusive. Given in 0.5 and 1 gram doses, both the free acid and its potassium salt produced strong symptoms of distress, but after a few hours the animals regained their normal appearance. Larger doses passed through the bowel in a very short time and no definite symptoms developed.

It is difficult to determine just what caused the toxicity of the preparations which were used in the experiments described by Crawford.[3] It is evident that very impure substances were given.

[1] Anderson, *Jour. Biol. Chem.* 11: 484 (1912) and Tech. Bull. No. 19, N. Y. Agr. Exp. Sta. (1912).

[2] Ibid, 12: 109 and 111 (1912) and Tech. Bull. No. 21, N. Y. Agr. Exp. Sta. (1912).

[3] Loc. cit.

It is our purpose to carry out a series of experiments to determine the toxicity of the acid from cottonseed meal in comparison with phytic acid.

EXPERIMENTAL.

The cottonseed meal used in these experiments was obtained from the stock used as cattle feed at this station. For the first preparation 4500 grams of meal were digested in 10 liters of 0.2 per ct. hydrochloric acid over night. It was then pressed through cheesecloth and the extract filtered through a layer of clean sand. The extract was a thick, mucilaginous, very dirty-colored liquid which could not be filtered through paper. It measured about 5 liters. It was mixed with about 8 liters of alcohol which produced a very fine and voluminous dirty precipitate. After settling over night the supernatant liquid was syphoned off and the residue centrifuged. The precepitate was then digested in a considerable quantity of 0.5 per ct. hydrochloric acid and the insoluble portion removed by centrifuging and the solution precipitated with excess of barium hydroxide. The mixture was heated nearly to boiling and then allowed to cool and settle. It was again centrifuged and the residue treated with 0.5 per ct. hydrochloric acid in which it was readily soluble. After a few minutes, however, it began to separate as a fine crystalline precipitate. The mixture was then filtered and the above precipitate reserved for special examination.

The filtrate was precipitated by the addition of alcohol, filtered, and again treated with 0.5 per ct. hydrochloric acid, filtered from insoluble matter and the filtrate again precipitated by alcohol. It was filtered and washed in dilute alcohol and then dissolved in 0.5 per ct. hydrochloric acid; heated nearly to boiling and filtered. The filtrate was now nearly colorless and it was slightly opalescent in appearance. After again precipitating the hydrochloric acid solution with alcohol the substance was obtained as a white amorphous powder. It was very soluble in 0.5 per ct. hydrochloric acid but the solution had a thick, mucilaginous and slightly opalescent appearance. This solution was now precipitated with excess of barium hydroxide when a voluminous, tenacious, ropy precipitate was obtained. The mixture was thoroughly shaken for some time and then filtered and washed in water. The washed residue was dissolved in 0.5 per ct. hydrochloric acid and precipitated with alcohol. After repeating this operation the substance was filtered, washed free of chlorides with dilute alcohol and finally washed in alcohol and ether and dried in vacuum over sulphuric acid. The product was then a snow-white amorphous powder and it weighed 10.2 grams.

It was but slightly soluble in boiling water. With phloroglucine and hydrochloric acid it gives a light red color which soon changes to a reddish-brown. With orcine it gives at first a reddish color which soon fades leaving a dirty-colored precipitate. After boiling

the substance in dilute hydrochloric acid, precipitating the barium with sulphuric acid, filtering and neutralizing, it strongly reduces Fehling's solution on boiling. The nitric acid solution gave no reaction with ammonium molybdate but after continued heating a slight precipitate was obtained. The substance was free from nitrogen and sulphur.

After drying at 105° in vacuum over phosphorus pentoxide it was analyzed.

0.2925 gram subst. gave 0.0894 gm. H_2O and 0.2338 gm. CO_2

0.2514 gram subst. gave 0.0972 gm. $BaSO_4$ and 0.0933 gm. $Mg_2P_2O_7$.

Found C = 21.80; H = 3.42; P = 10.34; Ba = 22.75 per ct.

While the substance was very slightly soluble in boiling water it was found when it was rubbed up in a mortar with a small quantity of cold water that it quickly dissolved but it began soon to separate again. Under the microscope the precipitate was seen to consist of small balls or globular masses of very fine microscopic needles.

Four grams of the substance were treated as mentioned above. After standing for two days at room temperature the crystalline precipitate was filtered off, washed in water, alcohol and ether and dried in the air. The snow-white crystalline powder was analyzed after previously drying at 105° in vacuum over phosphorus pentoxide.

0.2092 gram subst. lost 0.0291 gm. H_2O

0.1801 gram subst. gave 0.0232 gm. H_2O and 0.0379 gm. CO_2

0.1754 gram subst. gave 0.1262 gm. $BaSO_4$ and 0.0965 gm. $Mg_2P_2O_7$.

Found C = 5.73; H = 1.44; P = 15.33; Ba = 42.34; H_2O = 13.91 per ct.

The composition of this product differs entirely from that of the starting material but it agrees closely with that required for tetrabarium phytate.

Calculated for tetrabarium phytate, $C_6H_{16}O_{27}P_6Ba_4 = 1255$.

C = 5.73; H = 1.27; P = 14.82; Ba = 43.74; $11H_2O$ = 13.62 per ct.

The filtrate from the above crystalline compound was precipitated by alcohol, filtered, washed and dried in vacuum over sulphuric acid. It was a perfectly white amorphous powder. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1758 gram subst. gave 0.0790 gm. H_2O and 0.2222 gm. CO_2

0.1247 gram subst. gave 0.0223 gm. $BaSO_4$ and 0.0201 gm. $Mg_2P_2O_7$.

Found C = 34.47; H = 5.02; P = 4.49; Ba = 10.52 per ct.

The substance was very soluble in 0.5 per ct. hydrochloric acid in which it gave the same thick, mucilaginous, slightly opalescent solution as mentioned above.

The compound first analyzed is evidently not homogeneous. It apparently consists of some carbohydrate or gummy substance and an organic phosphorus compound; the latter crystallizes from the aqueous solution in nearly pure form but the substance cannot be separated by precipitating the dilute acid solutions with alcohol. This gummy substance has not been isolated in pure form and we are entirely in the dark as to its nature and composition.

A portion of the above crystalline barium salt was used for the preparation of the free acid. The substance was, however, not pure and it had not been washed free of the mother liquor. The acid was prepared by the usual method, that is, the barium salt was decomposed with a slight excess of sulphuric acid, the filtered solution precipitated with copper acetate, the latter filtered, washed and decomposed with hydrogen sulphide, again filtered and the filtrate evaporated in vacuum at a temperature of 40–45° and finally dried in vacuum over sulphuric acid. In appearance and reactions the acid was practically identical with phytic acid except that after boiling with dilute hydrochloric acid and neutralizing it slightly reduced Fehling's solution. This reduction, however, we believe to be due to admixed impurities; for, as stated above, the acid was not prepared from a pure compound.

The aqueous solution of the acid coagulates egg albumen at once. As has been already mentioned phytic acid gives the same reaction as well as the inosite esters of phosphoric and pyrophosphoric acids. Apparently, therefore, no special significance can be attached to this reaction.

The acid gave the following result on analysis after previously drying at 105° in vacuum over phosphorus pentoxide:

Found C = 11.71; H = 3.07; P = 26.35 per ct.

The crystalline precipitate mentioned on page 6, which separated from the solution of the first barium precipitate in 0.5 per ct. hydrochloric acid, was treated with about 5 per ct. hydrochloric acid in which the greater portion dissolved. The insoluble matter was removed by centrifuging and the solution precipitated with alcohol. This operation was repeated a second time when the substance was obtained nearly white. It differed from the first preparation in that its solution in dilute hydrochloric acid was neither mucilaginous nor opalescent. For further purification the substance was first precipitated by barium hydroxide from its dilute hydrochloric acid solution, and then twice precipitated from dilute hydrochloric acid with alcohol. The precipitates produced by the alcohol were amorphous at first but when allowed to stand over night in the mother liquor they always changed to the same crystalline form as previously mentioned.

After precipitating the last time with alcohol the substance was quickly filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white amorphous powder and it weighed 7.4 grams.

The filtrate from the above was allowed to stand over night when a small amount of the substance crystallized out. The crystals were filtered, washed in dilute alcohol, alcohol and ether and dried in the air. The substance was free from chlorine and gave no appreciable color reaction with phloroglucine or orcine. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0.2450 gram subst. lost 0.0272 gm. H_2O on drying
0.2178 gram subst. gave 0.0284 gm. H_2O and 0.0522 gm. CO_2
0.1776 gram subst. gave 0.1170 gm. $BaSO_4$ and 0.1078 gm. $Mg_2P_2O_7$.

Found C = 6.53; H = 1.45; P = 16.91; Ba = 38.76; H_2O = 11.10 per ct.

This substance agrees nearly in composition with tribarium phytate.

Calculated for tribarium phytate, $C_6H_{18}O_{27}P_6Ba_3 = 1120$.

C = 6.42; H = 1.60; P = 16.60; Ba = 36.78; $8H_2O$ = 11.39 per ct.

The amorphous product (7.4 grams) mentioned above was analyzed after previous drying at 105° in vacuum over phosphorus pentoxide and the following results obtained:

C = 8.04; H = 1.62; P = 16.65; Ba = 36.55 per ct.

The substance was free from chlorine. It was very slightly soluble in boiling water. With phloroglucine it gave a cherry red color; with orcine only a faintly, greenish color was produced. After boiling in dilute hydrochloric acid, precipitating the barium with sulphuric acid, filtering and neutralizing, it reduced Fehling's solution slightly on boiling. Evidently some carbohydrate was still present.

For further purification the substance was dissolved in 0.5 per ct. hydrochloric acid, filtered and alcohol added until a faint permanent turbidity remained. This was cleared up by the addition of a few drops of dilute hydrochloric acid and the solution allowed to stand at room temperature. The substance soon began to separate in the same crystalline form as before. After standing for two days the crystalline substance was filtered off, washed in water, alcohol and ether and dried in vacuum over sulphuric acid.

The mother liquor was diluted with more alcohol and allowed to stand as before when a further quantity of the same shaped crystals was obtained. After filtering, washing and drying as before these salts were analyzed after first drying at 105°.

Found, first crop of crystals:

C = 7.06; H = 1.53; P = 16.46; Ba = 38.16 per ct.

Found, second crop of crystals:

C = 7.47; H = 1.58; P = 16.46; Ba = 38.12 per ct.

In order to determine if further treatment would alter the composition the whole substance was digested in 50 per ct. acetic acid over night, filtered, washed in water, alcohol and ether and dried in the desiccator. It was then dissolved in 0.5 per ct. hydrochloric acid, filtered and the solution brought to crystallization by the careful addition of alcohol as before. The product finally obtained weighed 3.8 grams and it was a snow-white crystalline powder. For analysis it was dried at 105° in vacuum over phosphorus pentoxide. Found: C = 7.10; H = 1.52; P = 17.17; Ba = 38.11 per ct. As continued treatment did not alter the composition and as it separated in crystalline form it was undoubtedly a homogeneous compound.

The free acid prepared from this purified barium salt by the same method as before gave the following result on analysis after previous drying at 78° in vacuum over phosphorus pentoxide:

0.2626 gram subst. gave 0.0763 gm. H_2O and 0.1049 gm. CO_2
0.1733 gram subst. gave 0.1686 gm. $Mg_2 P_2 O_7$.
Found C = 10.89; H = 3.25; P = 27.11 per ct.
Calculated for phytic acid $C_6H_{24}O_{27}P_6$ = 714.
C = 10.08; H = 3.36; P = 26.05 per ct.

This acid gave the same reactions as previously described.

PREPARATION OF INOSITE FROM THE ABOVE BARIUM SALT.

Of the dry salt, 1.34 grams were heated in a sealed tube with 10 cc. 5/N sulphuric acid to 160° for about three hours. After precipitating with barium hydroxide, the inosite was isolated in the usual way and recrystallized from dilute alcohol with addition of ether. The product was obtained in colorless needles free from water of crystallization. The yield was 0.17 grams or about 77 per ct. of the theory. It gave the reaction of Scherer and melted at 221° (uncorrected). The air dried substance was analyzed:

Found C = 39.81; H = 6.96 per ct.

A further quantity of the barium salt was prepared by the following method, which was found to be much less laborious than that used at first. The cottonseed meal, 8 kilograms, was digested in 16 liters of 0.2 per ct. hydrochloric acid for about 5 hours. It was then pressed through cheesecloth and the extract filtered through absorbent cotton. The extract was precipitated with excess of barium hydroxide, allowed to settle and then centrifuged. The precipitate was digested in several liters of 0.5 per ct. hydrochloric acid and again centrifuged. The free acid was then nearly neutralized with barium hydroxide. The precipitate which separated was the barium salt of the organic phosphoric acid. This was filtered and treated with 0.5 per ct. hydrochloric acid, in which it was readily soluble at first, but it soon separated in the usual crystal aggregates. This was filtered and washed and dissolved in sufficient dilute hydrochloric acid and again filtered. The practically colorless filtrate was precipitated by alcohol. After filtering, it was again dissolved in dilute hydrochloric acid and precipitated with barium hydroxide, filtered and washed in water. It was then dissolved in dilute hydrochloric acid, precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white amorphous powder, and it weighed 24 grams. It was dissolved in about 300 cc. 0.5 per ct. hydrochloric acid filtered and allowed to stand a short time, when a portion crystallized out. This was filtered off, washed several times in water and finally in alcohol and ether, and dried in the air. The white crystalline powder weighed 7.4 grams. The filtrate and

washings from above were united and precipitated by alcohol. After standing over night the amorphous precipitate had changed to the usual crystalline form. After filtering, washing and drying in vacuum over sulphuric acid it weighed 14.7 grams.

The above salts were free from chlorine. The nitric acid solutions gave no immediate reaction with ammonium molybdate. No appreciable color reactions were obtained with phloroglucine or orcine and they did not reduce Fehling's solution. Metals other than barium were absent. For analysis the substances were dried at 105° in vacuum over phosphorus pentoxide.

The first crystalline compound gave the following: C = 6.05; H = 1.45; P = 16.51; Ba = 40.04; H_2O = 12.06 per ct. This salt is evidently a mixture of the tri- and tetrabarium salt. It was recrystallized as follows: One gram of the substance was dissolved in about 150 cc. of 0.5 per ct. hydrochloric acid and the free acid nearly neutralized with barium hydroxide. About 0.5 gram of barium chloride was then added and the solution allowed to stand for two days at room temperature. The substance separated slowly in the same general crystal form as before, except that the individual crystals were much larger. These were filtered, washed in water, alcohol and ether and dried in the air. Under the microscope the substance appeared homogeneous. Yield 0.9 grams. After drying at 105° in vacuum it was analyzed:

0.4067 grams subst. lost 0.0496 grams on drying.
0.3571 grams subst. gave 0.0358 grams H_2O and 0.0699 grams CO_2.
0.2068 grams subst. gave 0.1530 grams Ba SO_4 and 0.1160 grams $Mg_2P_2O_7$.

Found: C = 5.33; H = 1.12; P = 15.63; Ba = 43.53; H_2O = 12.19 per ct.

Calculated for tetrabarium phytate $C_6H_{16}O_{27}P_6Ba_4$ = 1255.
C = 5.73; H = 1.27; P = 14.82; Ba = 43.74; 10 H_2O = 12.54 per ct.

The second crystalline compound mentioned above gave the following result on analysis:
C = 6.88; H = 1.50; P = 15.94; Ba = 37.38 per ct.
P = 16.28; Ba = 37.21 per ct.
This corresponds to a tribarium salt.

A further quantity of the free acid was prepared from this salt by the usual method. From 7 grams of the substance practically the theoretical quantity of acid was obtained. After drying at 100° the substance was analyzed:

0.2319 grams subst. gave 0.0767 grams H_2O and 0.0925 grams CO_2.

After drying over boiling chloroform over phosphorus pentoxide:
0.2378 grams subst. gave 0.0703 grams H_2O and 0.0961 grams CO_2.
0.1495 grams subst. gave 0.1414 grams $Mg_2 P_2 O_7$.
Found C = 10.87; H = 3.70 per ct.
C = 11.02; H = 3.30; P = 26.36 per ct.

Calculated for phytic acid, $C_6H_{24}O_{27}P_6 = 714$.
C = 10.08; H = 3.36; P = 26.05 per ct.

This preparation gave the same reactions as those previously mentioned. When carefully prepared the acid is a thick colorless syrup readily soluble in water and alcohol. Attempts were made to prepare crystalline salts of the acid with organic bases like pyridine and brucine, but without success. These salts could not be obtained in crystalline form. In every case they separated as thick liquids, which could not be brought to crystallize even after long standing.

The reactions of the aqueous solution of the acid with inorganic bases may be briefly stated as follows:

It is not precipitated by chlorides of the alkaline earths, but acetates and hydroxides produce white amorphous precipitates. Ammonium molybdate gives a heavy white crystalline precipitate. Silver nitrate in excess produces a heavy white amorphous precipitate. Magnesia mixture also gives a voluminous white amorphous precipitate. While barium chloride does not give any precipitate, if the solution is allowed to stand at room temperature over night or longer the barium salt crystallizes out in delicate needle-shaped crystals. In shape and arrangement these crystals are identical with those previously referred to but they are much larger. It immediately coagulates egg albumen.

A neutral solution of the acid does not reduce Fehling's solution: even after boiling with dilute hydrochloric acid for some time no reduction takes place. No appreciable color reaction is given with phloroglucine or orcine.

Ferric chloride gives a white precipitate very sparingly soluble in hydrochloric acid. Copper acetate in excess gives a bluish-white precipitate.

On drying at 78° or 100° the substance turns very dark in color, but on drying at 60° the color changes but slightly. All the barium salts obtained were strongly acid in reaction on moist litmus paper.

It is evident that the substance isolated from cottonseed meal is very similar to phytin. The various salts which have been analyzed show but little difference in composition as compared with the corresponding phytin derivatives. It may be noted, however, that the analytical results of the purified, so-called, tri-barium salts point to the empirical formula $C_2 H_4 P_2 O_8 Ba$. Such a compound might be a monobasic acid of the formula $CH_3 PO_4$, but it is also isomeric with inosite hexaphosphate and accordingly differs very little in composition from phytic acid.

Whether the organic phosphoric acid in cottonseed meal is identical with phytin, an isomer, or is a somewhat differently constituted substance, can hardly be determined from the data presented in this paper. The investigation will be continued.

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New York Agricultural Experiment Station.

GENEVA, N. Y.

COMPOSITION AND PROPERTIES OF SOME CASEIN AND PARACASEIN COMPOUNDS AND THEIR RELATIONS TO CHEESE.

LUCIUS L. VAN SLYKE AND ALFRED W. BOSWORTH.

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TECHNICAL BULLETIN No. 26.

COMPOSITION AND PROPERTIES OF SOME CASEIN AND PARACASEIN COMPOUNDS AND THEIR RELATION TO CHEESE.

LUCIUS L. VAN SLYKE AND ALFRED W. BOSWORTH.

SUMMARY.

1. Object.— The work was undertaken to obtain more information regarding the compounds formed by casein and paracasein with bases, especially with Ca. Two compounds have been previously prepared, one neutral to phenolphthaleïn, containing 1.78 per ct. Ca (2.50 CaO), and the other, neutral to litmus, containing 1.07 per ct. Ca (1.50 CaO). Our main object was to learn if there were other compounds containing less Ca. Another purpose was to ascertain the composition of the substance formed in cheese which is insoluble in water but soluble in 5 per ct. solution of Na Cl.

2. Method of preparing casein.— Casein must be made base-free for use in such work. Preparations were made containing less than 0.1 per ct. of ash. The usual method was employed in part, precipitating separator skim-milk with dilute acetic acid, redissolving the washed precipitate in dilute NH_4OH, continuing precipitation and solution three or more times. Finally, the remaining calcium is precipitated from the ammonia solution as oxalate, the precipitate being removed by centrifuging and filtering, and the filtrate precipitated with dilute HCl. After washing free from HCl, the casein is treated with alcohol and ether, and after grinding and partial drying is dried over H_2SO_4 under reduced pressure. Analysis of such casein preparations agrees with the composition generally accepted, except in the amount of phosphorus and sulphur.

3. Preparation and composition of basic calcium caseinate.— The compound was prepared in two ways, (1) by decomposing $CaCO_3$ with casein and (2) by treating casein with a lime-water solution and neutralizing the excess with HCl, with phenolphthaleïn as indicator. The composition of the resulting compound was determined (1) by weighing the CO_2 expelled from $CaCO_3$, (2) by determining the Ca in the resulting casein compound and (3) by analysis of compound formed by treating lime-water solution of casein with acid until neutral to phenolphthaleïn. The different results agree closely, showing basic calcium caseinate to contain about 1.78 per ct. Ca (2.50 CaO), or 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca.

(4) Acid or unsaturated caseinates of ammonium, sodium and potassium.— These compounds were prepared as follows: Ash-free casein is dissolved in alkali so that 50 cc. of $\frac{N}{50}$ alkali contain 1 gram of casein. This is neutralized with $\frac{N}{50}$ HCl, which is added in small portions, under constant agitation, until a permanent precipitate begins to appear, as shown by centrifuging a portion of the mixture in a sedimentation tube. This method enables one to detect the casein precipitated by 0.20 cc. of $\frac{N}{50}$ HCl. The point at which a permanent precipitate first begins to appear is noted and addition of acid is continued until all the casein is precipitated, which point is also noted. Three different casein preparations were used and numerous determinations were made. It was found that 1 gram of casein forms a soluble compound with each of the alkalis used when combined with amounts somewhere between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents of alkali; or, 1 cc. of $\frac{N}{10}$ alkali combines with an amount of casein somewhere between 0.87 and 0.91 gram. The proportion of basic element in each compound is as follows: NH_4, 0.20 per ct.; Na, 0.26 per ct.; and K, 0.44 per ct. Such casein compounds contain the smallest known amount of base and it is suggested that they be called mono-basic caseinates.

Special preparations were made of mono-ammonium caseinate, the compound being isolated and prepared in dry form. This was found to have the composition called for by the previous results obtained with the volumetric work.

(5) Acid or unsaturated caseinates of calcium, strontium and barium.— When a solution of casein in a hydroxide of calcium, etc., is treated with an acid, the caseinate is precipitated by the chloride formed; this difficulty can be overcome by removal of the chloride through simple dialysis before the amount is sufficient to cause precipitation. One gram of ash-free casein is dissolved in 250 cc. of $\frac{N}{50}$ hydroxide solution and $\frac{N}{50}$ HCl is added until the first sign of a permanent precipitate appears, as shown by centrifuging a portion. The solution is then dialyzed to remove soluble chloride and then acid is again added until precipitation again occurs and another dialysis is made. Alternate addition of acid and dialysis are continued until finally the dialyzed solution forms a permanent precipitate with the addition of any acid. The results of many experiments agree in indicating the formation of two sets of compounds, mono-basic and di-basic, one set containing twice as much base as the other. In the di-basic compounds, 1 gram of casein requires between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents of hydroxide to form a compound soluble in water but easily precipitable by even a small amount of a soluble chloride of calcium, strontium or barium. In the di-basic compounds, 1 gram of casein combines (a) with 0.44 to 0.46 gram Ca (0.62 to 0.64 CaO), (b) with 0.96 to 1.01 gram Sr (1.14 to 1.19 SrO), and (c) with 1.51 to 1.58 grams Ba (1.69 to 1.76 BaO). In the mono-basic salts, 1 gram of

casein combines with about 1.1 x 10^{-4} gram equivalents of hydroxides to form *insoluble* compounds, which are soluble in 5 per ct. solution of chloride of sodium, ammonium or potassium. This solubility is due to an exchange of bases; for example, insoluble mono-calcium caseinate is changed by treatment with solution of NaCl into soluble mono-sodium caseinate and $CaCl_2$, as shown by special experiments.

Special preparations were made of mono- and di-calcium caseinates, each compound being isolated and prepared in dry form. These were found to have essentially the composition called for by the previous results obtained with the volumetric work.

(6) Valency of casein molecule and molecular weight of casein.— On the basis of the composition of the basic calcium caseinate and mono-calcium caseinate, the former has a valency of 8. These relations indicate the molecular weight of casein to be 8888 and the equivalent weight 1111.

(7) Method of preparing paracasein.— Separator skim-milk is heated to 37° C. and treated with 0.12 cc. of rennet-extract (Hansen's) per 1,000 cc. of milk. The milk is allowed to stand until completely precipitated. The resulting curd is broken up by vigorous stirring, the whey removed and the precipitated paracasein washed freely with water. It is then dissolved in dilute NH_4OH, reprecipitated with acid and the operation continued and completed as in case of casein.

(8) Preparation and composition of basic calcium paracaseinate.— By the same methods of study, paracasein was shown to form with calcium a paracaseinate similar in composition and properties to that of basic calcium caseinate.

(9) Acid or unsaturated paracaseinates of ammonium, sodium and potassium.— In these compounds, 1 gram of paracasein combines with an amount of alkali somewhere between 2.2 x 10^{-4} and 2.3 x 10^{-4} gram equivalents in forming soluble compounds with ammonium, sodium and potassium, which are acid to both litmus and phenolphthalein. One cc. of alkali combines with 0.435 to 0.455 gram of paracasein. The percentage of basic element in each compound is, NH_4 0.40; Na, 0.52; and K, 0.88. The amount of each basic element in these paracaseinates is just twice that present in the corresponding casein compounds.

A preparation of mono-ammonium paracaseinate in dry form gave results agreeing fairly well in composition with the results obtained by volumetric work.

(10) Acid or unsaturated paracaseinates of calcium, strontium and barium.— Mono- and di-basic paracaseinates were prepared in the same manner as the corresponding caseinates and were shown to differ from them in having just twice as much of the basic element. In the mono-basic compounds, which are *insoluble*, 1 gram of paracasein combines with about 2.3 x 10^{-4} gram equivalents of hydroxide of calcium, etc.; in the di-basic, which are *soluble*, with about

4.6 x 10^{-4} gram equivalents. Their properties resemble those of the corresponding caseinates.

(11) Valency of paracasein molecule and molecular weight of paracasein.— The valency and molecular weight are shown to be one-half those of casein.

(12) Action of rennet-enzym on casein in forming paracasein.— When casein is treated with rennet-enzym, the casein molecule appears to be split into two molecules of paracasein.

(13) Composition of brine-soluble compound in cheese.— During the manufacture and ripening of cheddar and many other kinds of cheese, a protein is always formed which is insoluble in water but soluble in a 5 per ct. solution of NaCl. Former studies led to erroneous conclusions regarding its identity. Extended study shows that this substance is identical with mono-calcium paracaseinate.

INTRODUCTION.

The uncombined protein, casein, shows the characteristic property of an acid in combining with bases of the alkalis and alkaline earths to form salts and in decomposing their carbonates. The compounds thus formed, especially those with calcium, have been studied by numerous investigators, the more important contributions have been made by the following:

Hammarsten (Zur Kenntniss des Kaseins etc., Upsala, 1877);
Söldner (*Landw. Versuchs.-Stat.*, **35:** 351, 1888);
Courant (*Pflüger's Arch. Physiol.*, **50:** 109, 1891);
Timpe (*Arch. Hyg.*, **18:** 1, 1893);
Béchamp (*Bull. Soc. Chim.* (3) **11:**, 152, 1894);
de Jager (*Maly Jahresber. Thierchem.*, **27:** 276, 1897);
Salkowski (*Zeitschr. Biol.*, **37:** 415, 1899);
Kobrak (*Pflüger's Arch. Physiol.*, **80:** 69, 1900);
Osborne (*Jour. Physiol.*, **27:** 398, 1901);
Laqueur and Sackur (*Beitr. Chem. Physiol. u. Pathol.*, **3:** 193, 1902);
Van Slyke and Hart (Bull. No. 261, N. Y. Agr. Exp. Sta., and *Am. Chem. Jour.*, **33:** 472, 1905);
Long (*Jour. Am. Chem. Soc.*, **28:** 72, 1906);
Robertson (*Jour. Biol. Chem.*, **2:** 317, 1906);
Robertson (*Jour. Physic. Chem.*, **13:** 469, 1909).

Without going into details, it is sufficient for our purpose at this point to state that results have been reported in which compounds, formed by treating casein with calcium hydroxide, contain an equivalent of calcium oxide varying all the way from 0.8 to 3 per ct. (equal to 0.57 to 2.14 per ct. of calcium).

In the chemical laboratory of this Station, the relation of casein and paracasein to bases has been a subject of continued study for several years, especially in connection with changes taking place in the operation of cheese-making. The results here presented include

a careful revision of work published in previous bulletins of this laboratory with material extensions of the line of investigation.

Two compounds of casein and calcium have been generally recognized, one containing about 2.50 per ct. CaO (1.78 per ct. Ca), and the other about 1.50 per ct. CaO (1.07 per ct. Ca). We have found, in addition, two others, one containing about 0.31 per ct. CaO (0.22 per ct. Ca), and the other double this amount. Corresponding compounds are shown in our work to be formed by paracasein with calcium, and also by both casein and paracasein with ammonium, sodium, potassium, barium and strontium. A study of the methods of preparation and of the properties of these compounds is given in this publication; the ground covered is embraced under the following outline:

Part I. Casein and some of its compounds.
1. Method of preparing ash-free casein.
2. Preparation and composition of basic calcium caseinate.
3. Preparation and composition of unsaturated or acid caseinates.
4. Valency of casein molecule and the molecular weight of casein.

Part II. Paracasein and some of its compounds.
1. Method of preparing ash-free paracasein.
2. Preparation and composition of basic calcium paracaseinate.
3. Preparation and composition of unsaturated or acid paracaseinates.
4. Valency of paracasein molecule and the molecular weight of paracasein.
5. Action of rennet-enzym on casein.

Part III. Composition of brine-soluble compound in cheese.
1. Brine-soluble compound formed in cheese-making.
2. Identity of brine-soluble compound and monocalcium paracaseinate.

PART I. CASEIN AND SOME OF ITS COMPOUNDS.

METHOD OF PREPARING ASH-FREE CASEIN.

Casein that is to be used in studying its relation to mineral bases must be free from all such bases. The preparation of really ash-free casein is much more difficult than has been commonly assumed. The so-called chemically-pure casein furnished by chemical-supply houses usually contains 0.6 per ct. of ash. The preparations used in various investigations in which the ash content has been reported rarely contain less than 0.2 per ct. of ash and not infrequently as much as 0.6 per ct.

The principal basic element in casein preparations, as usually made, is calcium. The calcium in casein preparations is usually due to the presence of a compound of calcium and casein, containing 0.22 per ct. Ca (equal to 0.31 per ct. CaO), as we shall show later. This salt is insoluble in water but easily soluble in a 5 per ct. solution of sodium chloride, while base-free casein is insoluble in both water and the brine solution.

When casein is carefully precipitated by dilute acids from milk or from lime-water solutions of casein, the precipitate is apt to contain more or less of the above-mentioned calcium caseinate as well as base-free casein. The precipitation of this calcium salt occurs most readily when the usual precautions in precipitating casein from milk are most rigidly observed, that is, when excess of acid is avoided. We have examined casein preparations obtained from chemical-supply houses and have found that some of them are soluble in a 5 per ct. solution of sodium chloride to the extent of 50 per ct., or more, of their weight.

After trying different methods of preparing casein so as to contain a minimum amount of calcium, we have obtained the most satisfactory results by the method described below. We have been able to prepare casein containing only 0.06 per ct. of ash, consisting largely of calcium phosphate, derived from the trace of calcium not removed and the phosphorus of the casein molecule. The amount of calcium present in 5 grams of such material was too small to determine quantitatively.

Our method of preparation is to dilute separator skim-milk with seven or eight times its volume of distilled water and carefully add dilute acetic acid (6 cc. of glacial acetic acid diluted to 1 liter) until the casein separates completely, after which the clear solution is removed by siphon as soon as the precipitate settles. Distilled water is then added, the mixture stirred vigorously and the precipitate allowed to settle, after which the wash-water is siphoned off. More water is then added and the casein is dissolved by adding, for each liter of milk used, 1 liter of dilute ammonium hydroxide (6 cc. of strong reagent diluted to 1 liter). When the solution is complete, the whole is filtered through a thick layer of absorbent

cotton. The casein is then precipitated again with dilute acetic acid; the precipitate is allowed to settle, and is then washed, redissolved in dilute ammonium hydroxide, and filtered, the process of precipitation, washing, dissolving, etc., being repeated not less than four times. Finally an excess of strong ammonium hydroxide (10 cc.) is added and then 20 cc. of saturated solution of ammonium oxalate. The mixture is allowed to stand 12 hours or more. Calcium is precipitated as oxalate in very finely divided condition, too fine to permit its satisfactory removal by ordinary methods of filtration. Better aggregation of the precipitate can, however, be effected by means of centrifugal force. The centrifuged mixture is then filtered through double thickness of filter paper. The filtered solution is next treated with dilute hydrochloric acid (10 cc. of HCl, sp. gr. 1.20, diluted to 1 liter) until the casein is precipitated. The precipitate is washed with distilled water until free from chloride and is then placed on a hardened filter paper in a Buchner funnel, as much water as possible being now removed from the precipitate by suction. The mass is next transferred to a large mortar and thoroughly triturated with 95 per ct. alcohol. The alcohol is then removed by suction on a Buchner funnel and the casein is then again placed in a mortar and triturated with absolute alcohol. Most of the alcohol is removed by filtration and the casein treated twice with ether in a mortar by trituration, the ether being removed each time by means of suction on a Buchner funnel. The material is then placed in a large evaporating dish and spread out in a layer as thin as possible; it is allowed to stand 12 hours or more in a warm place; and is finally ground in a mortar until the particles pass a 40-mesh sieve, and is dried two days over sulphuric acid in a desiccator under diminished pressure.

Three preparations made in this way were found to show an ash content of 0.10, 0.09 and 0.06 per ct., respectively. These preparations were insoluble in water and in 50 per ct. alcohol; the first one was very slightly soluble in a 5 per ct. solution of sodium chloride, but the two others were not.

When one gram of these casein preparations was treated with 10 cc. of $\frac{N}{10}$ hydroxide of ammonium, sodium or potassium, and 90 cc. of water, a clear solution was obtained, the casein dissolving completely. When to this solution a minute amount of a solution of a barium, calcium or strontium salt was added, there developed promptly the opalescent appearance characteristic of casein solutions under such conditions.

Casein prepared in the manner described was analyzed, with the following results:

	Per ct.		*Per ct.*
Moisture	1.09	In dry substance:	
In dry substance:		Nitrogen	15.80
Ash	0.06	Phosphorus	0.71
Carbon	53.50	Sulphur	0.72
Hydrogen	7.13	Oxygen (by difference)	22.08

PREPARATION AND COMPOSITION OF BASIC CALCIUM CASEINATE.

The compound commonly known as basic calcium caseinate contains the largest amount of calcium in combination with casein. This is the compound that has been most frequently prepared and studied by investigators, beginning with Söldner (see references on page 6). Varying results have been obtained by different workers, the percentage of calcium ranging from 1.66 to 2.13 per ct. (equivalent to 2.32 to 2.98 per ct. CaO).

This compound can be prepared in two different ways: (1) By decomposing calcium carbonate with casein and (2) by treating casein with a solution of calcium hydroxide (lime-water).

Preparation of basic calcium caseinate by treating casein with calcium carbonate.— When casein is treated with calcium carbonate, the results of the reaction can be measured in two ways: (a) By weighing the carbon dioxide displaced, and (b) by determining the amount of calcium in the resulting compound. Both methods were used by us.

Casein prepared in the manner previously described was placed in the flask of a Knorr carbon dioxide apparatus and an excess of calcium carbonate suspended in water was added. The carbon dioxide formed in the reaction was run into weighed bulbs containing potassium hydroxide and the increase of weight due to carbon dioxide determined at the end of the reaction. The results are given in the following table.

TABLE I.— AMOUNTS OF CARBON DIOXIDE EXPELLED FROM CALCIUM CARBONATE BY CASEIN.

Amount of dry casein used.	Amount of CO_2 expelled.	Amount of Ca O (and Ca) for 100 grams of casein, equivalent to CO_2.
Grams.	*Grams.*	*Grams.*
10	0.1900	2.42 (1.73 Ca)
10	0.1980	2.52 (1.80 Ca)
5	0.1054	2.68 (1.91 Ca)
5	0.1003	2.55 (1.81 Ca)
Average	———	2.54 (1.81 Ca)

For the purpose of measuring the results of the reaction by determining the amount of calcium in the resulting compound, the casein was put in a mortar and thoroughly triturated with an excess of moist calcium carbonate, the excess being removed by filtration at the end of the reaction. The filtrate was treated with 95 per ct.

alcohol, which was free from acid, until the calcium caseinate was precipitated, after which the precipitate was washed with alcohol and ether, and dried at 120° C. A weighed portion of this compound was carefully ignited and the calcium in the resulting ash was determined, with the following results:

TABLE II.— AMOUNT OF Ca COMBINING WITH CASEIN WHEN REACTING WITH $CaCO_3$.

Weight of caseinate.	Weight of CaO.	Weight of Ca.	Weight of free casein.	CaO (and Ca) for 100 grams of casein.
Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*
0.4125	0.0102	0.0073	0.4052	2.52 (1.80 Ca)
0.5134	0.0124	0.0089	0.5045	2.46 (1.76 Ca)
0.3090	0.0077	0.0055	0.3035	2.54 (1.81 Ca)
0.4253	0.0104	0.0074	0.4179	2.49 (1.77 Ca)
Ave. 0.41505	0.010175	0.00726	0.4078	2.50 (1.78 Ca)

Preparation of basic calcium caseinate by treating casein with an excess of calcium hydroxide.— Weighed portions of casein were dissolved in an excess of lime-water. Phenolphthaleïn indicator was then added to the solution and hydrochloric acid was run in until the solution became neutral. The solution was then dialyzed to remove the calcium chloride formed in neutralization. The dialyzed solution was evaporated to dryness, the residue dried at 120° C. and weighed. The determination of calcium was made after ignition, with the following results:

TABLE III.— AMOUNT OF CALCIUM COMBINING WITH CASEIN ON TREATMENT WITH CALCIUM HYDROXIDE.

Weight of caseinate.	Weight of CaO.	Weight of Ca.	Weight of free casein.	CaO (and Ca) for 100 grams of casein.
Grams.	*Grams.*	*Grams.*	*Grams.*	*Grams.*
1.582	0.040	0.0286	1.5534	2.58 (1.84 Ca)
1.471	0.035	0.0250	1.4460	2.42 (1.73 Ca)
1.548	0.038	0.0271	1 5209	2.50 (1.78 Ca)
Ave. 1.534	0.0377	0.0269	1.5070	2.50 (1.78 Ca)

The three sets of figures presented in Tables I, II, and III indicate that casein combines with calcium to form a compound containing about 2.50 per ct. CaO (equal to 1.78 per ct. Ca); the compound in

solution is neutral to phenolphthaleïn. Expressed in another form, 1 gram of casein combines with 9×10^{-4} gram equivalents of calcium. This compound is commonly known as *basic calcium caseinate.*

PREPARATION AND COMPOSITION OF UNSATURATED OR ACID CASEINATES.

Compounds of casein with bases, in which less base is present than in the basic calcium caseinate described above, have been reported. Söldner[1] obtained a compound of casein and calcium containing 1.11 per ct. Ca (equal to 1.55 per ct. CaO); or, expressed in another form, 1 gram of casein combines with 5.55×10^{-4} gram equivalents of calcium. This compound is neutral to litmus but acid to phenolphthaleïn, and has been commonly known as *neutral calcium caseinate.* This compound as prepared by Van Slyke and Hart[2] contains about 1.07 per ct. Ca (equal to about 1.50 per ct. CaO), or 1 gram of casein combines with 5.35×10^{-4} gram equivalents of calcium. Courant[3] believes that, in addition to the basic and neutral compounds of casein and calcium, a third exists, in which the calcium is present in about one-half the amount contained in the neutral compound and one-third that contained in the basic compound; he regards them as mono-, di- and tri-calcium caseinates. Timpe[4] reports a compound containing 0.961 per ct. Na (equal to 0.868 per ct. CaO or 0.62 per ct. Ca; or 1 gram of casein combines with 3.1×10^{-4} gram equivalents of calcium). Long[5] was able to dissolve 1 gram of casein in just one-half the amount of alkali required for the phenolphthaleïn neutralization, and therefore inferred the existence of acid caseinates containing one-half the amount of base contained in basic calcium caseinate. The existence of such a combination is questioned by Robertson.[6]

In the course of our work, we became convinced that casein forms compounds containing less base than any of those reported by other workers. While we were at work on this point, an article by Robertson[7] appeared, in which was reported a combination of casein and sodium hydroxide, 1 cc. of the alkali combining with 0.877 gram of casein. Our further work confirms Robertson's results, although we have used a different method of procedure. In addition, we have been able to prepare and isolate several salts for analysis. Our study of these individual salts shows that ammonium, sodium and potassium compounds possess properties of solubility very different from those of barium, calcium and strontium. As previously stated,

[1] *Landw. Versuchs.-Stat.*, 35: 351, 1888.
[2] N. Y. Agrl. Expt. Sta. Bull. No. 261, 1905.
[3] *Pflüger's Archiv. Physiol.*, 50: 109, 1891.
[4] *Arch. Hyg.* 18: 1, 1893.
[5] *Jour. Am. Chem. Soc.*, 28: 372, 1906.
[6] *Jour. Biol. Chem.*, 2: 336, 1906.
[7] *Jour. Physical Chem.*, 13: 469, 1909.

we have prepared and studied two sets of compounds of casein with bases, in one of which 1 gram of casein combines approximately with 1.125×10^{-4} gram equivalents of base, while in the other 1 gram of casein combines with about 2.25×10^{-4} gram equivalents of base.

We will next take up the details of our experimental work in preparing acid caseinates of the bases of the more common alkalis and alkaline earths.

The specific object of our work was to ascertain the smallest quantity of base with which casein combines to form a definite salt. In the volumetric work our method of procedure was as follows: In 200 cc. of $\frac{N}{40}$ alkali, we dissolved 5 grams of pure casein as quickly as possible and then made the volume to 250 cc. Each 50 cc. of this solution therefore represents 1 gram of casein dissolved in 50 cc. of $\frac{N}{50}$ alkali. A preliminary or trial determination was next made in the following manner: Into a 300 cc. Erlenmeyer flask, we measure 50 cc. of the caseinate solution and then add, a drop at a time, some $\frac{N}{50}$ HCl, until we have used 5 cc., the contents of the flask being kept in constant agitation in order to prevent premature precipitation of casein. After addition of the 5 cc. of acid, a portion of the contents of the flask is centrifuged, in order to cause the sedimentation of precipitated casein, if any, a precipitate serving as an indicator. A sedimentation tube of 50 cc. capacity can be used; the precipitate collects in the lower V-shaped portion. It is possible in this manner to detect the casein precipitated by 0.20 cc. of $\frac{N}{50}$ HCl. In case no casein is precipitated by the first addition of 5 cc. of acid, another equal amount of acid is added and a portion of the mixture centrifuged; the process of adding 5 cc. portions of acid and centrifuging is continued until a permanent precipitate of casein is obtained. This shows, within 5 cc. of $\frac{N}{50}$ HCl, how much acid is required to start definite precipitation of the casein. In order to ascertain the exact point more closely, another set of determinations is made, using 50 cc. of the caseinate solution and adding in the same cautious manner an amount of $\frac{N}{50}$ HCl which is 5 cc. less than the amount causing the first appearance of a permanent precipitate in the trial or preliminary determination. The acid is now added in small amounts with constant agitation of the mixture to prevent the premature separation of any precipitate, and centrifuged after the addition of each 0.25 cc. The point at which a permanent precipitate first appears is noted; the addition of acid is continued until all the casein is precipitated and this point is also noted. In our work this method of determination was repeated several times with each combination of casein and alkali and three different casein preparations were used in preparing each caseinate. We will now present the results of our experimental work in connection with the unsaturated or acid caseinates of, first, ammonium, sodium and potassium, and, second, barium, strontium and calcium.

Acid caseinates of ammonium, sodium and potassium.— In the manner described above, we made numerous determinations in the case of preparations of base-free casein dissolved in the hydroxide of ammonium, sodium and potassium, respectively. Tabulated below, we give the average results of many such determinations.:

TABLE IV.— RELATION OF ALKALI BASES TO CASEIN IN ACID CASEINATES.

Amount of casein used.	Kind of alkali used	Amount of $\frac{N}{50}$ alkali used.	Amount of $\frac{N}{50}$ HCl required to cause first sign of permanent precipitation.	Amount of alkali left combined with casein.		Amount of $\frac{N}{50}$ HCl required to precipitate all of the casein.
				$\frac{N}{50}$.	$\frac{N}{10}$.	
Gram.		*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*
1	NH_4 OH	50	Between 44.25 and 44.50	5.5 to 5.75	1.1 to 1.15	50
1	Na OH	50	" " " "	" "	" "	50
1	K O H	50	" " " "	" "	" "	50

The results in this table indicate that 1 gram of casein forms a soluble compound with ammonium, sodium and potassium, when combined with amounts of each somewhere between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents of alkali; or, expressed in another form, 1 cc. of $\frac{N}{10}$ alkali combines with an amount of casein somewhere between 0.87 and 0.91 gram. The proportion of basic element in each compound is approximately the following: NH_4, 0.20 per ct.; Na, 0.26 per ct.; and K, 0.44 per ct. Caseinates combining with the amount of alkali base indicated contain the smallest known amount of base, according to our present knowledge. It seems proper, therefore, to suggest that such compounds be called mono-basic caseinates.

Preparation of mono-ammonium caseinate.— It seemed desirable that we should carry the work somewhat farther and prepare one pure compound, at least, in dry form for study. The ammonium compound was chosen as the one offering least difficulty. The method of preparation was as follows: In 2 liters of distilled water containing 250 cc. of $\frac{N}{10}$ NH_4OH, 25 grams of base-free casein were dissolved. After solution was complete, we slowly added 125 cc. of $\frac{N}{10}$ HCl, care being taken to agitate the mixture during the addition of the acid, in order to prevent premature precipitation of any casein. There was next added very cautiously $\frac{N}{50}$ HCl until a permanent precipitate began to appear, as shown by centrifuging the mixture. The solution was then filtered and measured. The amount of $\frac{N}{50}$ HCl required to precipitate the casein completely was determined in an aliquot part. Then one-third of this amount was added to insure the presence of only mono-basic caseinate. Any precipitate formed was removed by filtration and the filtrate was dialyzed until the ammonium chloride that had been formed in the

reaction was completely removed. The resulting solution, containing mono-ammonium caseinate, was then precipitated by addition of acid-free alcohol. The precipitate was filtered, washed with acid-free alcohol and ether and dried at 120° C. In several preparations thus made the amount of ammonia was determined; the results are given in the following table:

TABLE V.— COMPOSITION OF MONO-AMMONIUM CASEINATE.

Amount of caseinate used.	Amount of $\frac{N}{10}$ NH_4OH found.	Relation of casein to NH_4OH in caseinate.	Percentage of NH_4 in caseinate.
Grams.	*Cc.*		
5.891	6.64	1 gram of casein to 1.127×10^{-4} grams equivalents	0.203
4.870	5.38	1 " " " 1.105×10^{-4} " "	0.200
*4.000	4.30	1 " " " 1.075×10^{-4} " "	0.194
*3.000	3.16	1 " " " 1.053×10^{-4} " "	0.190

*Preparations of caseinates made by Mr. O. B. Winter.

Acid caseinates of calcium, strontium and barium.— In making preparations of the caseinates of the alkaline earth bases, difficulty was experienced in obtaining concordant results. The trouble was finally found to be due to the presence of the chloride formed when the solution of the caseinate is treated with hydrochloric acid. Such chlorides tend to cause precipitation of the caseinates either by decreasing their solubility or, perhaps, by formation of double salts, consisting of the chloride in combination with the caseinate.[1] The difficulty of insolubility is readily overcome by removal of the chloride through simple dialysis before the amount is sufficient to cause precipitation. To accomplish this, we made use of the following process: In 200 cc. of $\frac{N}{40}$ hydroxide of calcium, strontium or barium, we dissolved 5 grams of casein and then diluted the solution to 250 cc. A preliminary or trial determination was made by adding $\frac{N}{50}$ HCl to 50 cc. of the caseinate solution in portions of 5 cc. at a time, agitating constantly and, after each addition, testing for the presence of a precipitate by centrifuging a portion, until a precipitate appeared, just as in the case of preparing alkaline caseinates (p. 13). Then to each of several flasks containing 50 cc. of the caseinate solution we added an amount of $\frac{N}{50}$ HCl that was 5 cc. less than the amount causing the first appearance of a permanent precipitate in the preliminary trial. The contents of the flask were then placed in dialyzing tubes and, by frequent changes of the surrounding water, most of the soluble chloride that had been formed was removed. The contents of one tube were then used for another

[1] Pfeiffer and Modelski, *Ztschr. Physiol. Chem.*, 81: 329, 1912.

preliminary test. An amount of acid less than that required to produce a precipitate in this second test was then added to all the tubes and the contents again dialyzed. This operation was continued in the manner indicated in the following table:

TABLE VI.— ILLUSTRATION OF METHOD USED IN PREPARING ACID CASEINATES OF CALCIUM, STRONTIUM AND BARIUM.

Amount of casein in solution.	Amount of $\frac{N}{50}$ hydroxide solution used.	Amount of $\frac{N}{50}$ HCl added.	Sign of precipitation.	
Gram.	*Cc.*	*Cc.*		
1	50	30	Precip.	First trial.
1	50	25	0	Dialyzed and used for next.
1	50	30	0	" " " " "
1	50	35	Precip.	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	" " " " "
1	50	35	0	
1	50	40	Precip.	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	" " " " "
1	50	35	0	" " " " "
1	50	36	0	
1	50	37	0	
1	50	38	Precip.	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	" " " " "
1	50	35	0	" " " " "
1	50	37	0	" " " " "
1	50	38	0	
1	50	39	Precip.	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	" " " " "
1	50	35	0	" " " " "
1	50	37	0	" " " " "
1	50	38	0	" " " " "
1	50	38.5	0	
1	50	39	Precip.	

In the manner described above, we have made numerous preparations of calcium, strontium and barium caseinates; the averages of many results are given in Table VII.

We have found that in adding $\frac{N}{50}$ HCl to 50 cc. of a caseinate solution containing 1 gram of casein dissolved in 50 cc. of $\frac{N}{50}$ solution of hydroxide of calcium, strontium or barium, it requires less than

50 cc. of acid to precipitate the casein completely; the exact amount is 44.5 cc. of $\frac{N}{50}$ HCl. The remaining amount of base, equal to 5.5 cc. of $\frac{N}{50}$ hydroxide, or 1.1 cc. of $\frac{N}{10}$ hydroxide, appears to be held in combination in the insoluble compound.

TABLE VII.— CASEINATES OF CALCIUM, STRONTIUM AND BARIUM.

Amount of casein used.	Kind of hydroxide used.	Amount of $\frac{N}{50}$ hydroxide used.	Amount of $\frac{N}{50}$ HCl required to cause first sign of permanent precipitate.	Amount of base combined with casein in solution.		Amount of $\frac{N}{50}$ HCl required to precipitate all casein.	Amount of base in precipitated casein.	
				$\frac{N}{50}$	$\frac{N}{10}$		$\frac{N}{50}$	$\frac{N}{10}$
Grams.		*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*
1	$Ca(OH)_2$	50	38.5 to 39	11 to 11.5	2.2 to 2.3	44.5	5.5	1.1
1	$Sr(OH)_2$	50	" " "	" " "	" " "	"	"	"
1	$Ba(OH)_2$	50	" " "	" " "	" " "	"	"	"

These results indicate the formation of two sets of compounds, when casein is dissolved in a hydroxide of calcium, strontium or barium and this solution is neutralized with acid under the conditions of our experiments. One set of compounds contains twice as much base as the other.

Attention is called to additional details in the following statements:

(1) In the di-basic compounds, as the results show, 1 gram of casein requires between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents of hydroxide of calcium, strontium or barium to form a compound which is soluble in water when there is not present any, or more than a trace of, soluble chloride of any of these elements. The addition of even a small amount of a soluble salt of any of these elements to a solution of any of these di-basic caseinates causes the formation of a precipitate.

(2) In these di-basic compounds, 100 grams of casein combine (a) with 0.44 to 0.46 gram Ca (equal to 0.62 to 0.64 gram CaO), (b) with 0.96 to 1.01 gram Sr (equal to 1.14 to 1.19 grams SrO), or (c) with 1.51 to 1.58 grams Ba (equal to 1.69 to 1.76 grams BaO).

(3) Apparently, with the treatment described above, 1 gram of casein combines with about 1.1×10^{-4} gram equivalents of the hydroxide of calcium, strontium or barium to form an *insoluble* compound, when an acid is added in amount just sufficient to precipitate the casein completely. These compounds are regarded as *mono-basic.*

(4) In these insoluble mono-basic compounds 100 grams of casein combine approximately (a) with 0.22 gram Ca (equal to 0.31 gram CaO), (b) with 0.48 gram Sr (equal to 0.57 gram SrO), or (c) with 0.76 gram Ba (equal to 0.85 gram BaO).

(5) These insoluble compounds possess some highly interesting properties; they are soluble in a 5 per ct. solution of sodium, potas-

sium or ammonium chloride. This solubility is due to an exchange of bases, which, for our purpose, can be represented by the following reversible reaction:

$$\underset{\text{(insoluble)}}{\text{Ca}\begin{matrix}\text{caseinate}\\\text{caseinate}\end{matrix}} + 2\ \text{NaCl} \rightleftharpoons \underset{\text{(soluble)}}{2\ \text{Na caseinate}} + \text{CaCl}_2$$

That the reaction is a reversible one is supported by the following experimental evidence: Mono-calcium caseinate was prepared and freed from soluble calcium salts by washing and dialysis. The compound was then dissolved in a 5 per ct. solution of calcium-free sodium chloride. That an interchange of bases had taken place was shown by the fact that when the caseinate brine solution was dialyzed, calcium was found in the solution outside the dialyzing tube. This brine solution of caseinate was then dialyzed until free from calcium and was then filtered. A solution of calcium chloride was then added to this dialyzed solution and at once a precipitate of calcium caseinate was produced. That this precipitate is a calcium salt can be shown in two ways: (1) By washing and dialyzing until free from soluble chloride and then igniting. Calcium is found in the ash. (2) By washing and dialyzing until free from soluble calcium, then redissolving in 5 per ct. solution of calcium-free sodium chloride and dialyzing. Calcium is found to dialyze out of this brine solution of caseinate.

There is another point of interest in connection with this compound which we will briefly refer to here but consider in more detail in the report of another investigation. When a small amount of acid is added to milk or is formed in milk by lactic fermentation, a substance separates on warming which is very stringy and which easily dissolves in a 5 per ct. solution of sodium chloride. This substance is probably mono-calcium caseinate.

Preparation of mono- and di-calcium caseinates.— In order to study the composition and properties of these compounds more fully, preparations of mono- and di-calcium caseinates were made. The following method was employed: In 800 cc. of $\frac{N}{20}$ $Ca(OH)_2$ there were dissolved 20 grams of base-free casein. To this solution was added 400 cc. of $\frac{N}{20}$ HCl; the solution was then dialyzed to remove most of the resulting calcium chloride. Then $\frac{N}{20}$ HCl was added very cautiously under constant agitation of the mixture until a permanent precipitate began to appear, as shown by centrifuge. The solution was then dialyzed again and then more acid was added until a precipitate once more began to form. Alternate dialysis and addition of acid were continued until no more acid could be added without causing a precipitate. The amount of acid necessary to precipitate all of the casein was next determined in an aliquot portion, and one-third of this amount of acid was then added. The

precipitated casein was filtered out and the filtrate was dialyzed. This solution contained di-calcium caseinate. The solution was divided, one portion being used for the preparation of the di-calcium caseinate and the other for the mono-calcium caseinate.

In completing the preparation of the di-calcium caseinate, the salt was precipitated by addition of acid-free alcohol, the precipitate being washed with acid-free alcohol and ether, and then dried at 120° C. The composition of this preparation is given in Table IX.

In preparing the mono-calcium caseinate, the solution of di-calcium caseinate was treated with enough acid to precipitate three-fourths of the casein. The resulting precipitate was filtered, washed with water, acid-free alcohol and ether and then dried at 120° C. The results in Table VIII show the amount of calcium found in the preparation.

TABLE VIII.— COMPOSITION OF MONO-CALCIUM CASEINATE PREPARATION.

Amount of compound used.	Amount of CaO found.	Percentage of CaO in compound.	Relation of casein to calcium in compound.
Grams.	*Gram.*		
5	0.0149 (0.0106 Ca)	0.298 (0.213 Ca)	1 gram of casein to 1.06 x 10^{-4} gram equivalents.
5	0.0141 (0.0101 Ca)	0.282 (0.201 Ca)	1 gram of casein to 1.01 x 10^{-4} gram equivalents.
5	0.0146 (0.0104 Ca)	0.292 (0.209 Ca)	1 gram of casein to 1.04 x 10^{-4} gram equivalents.
Average...	0.01453 (0.0104 Ca)	0.291 (0.208 Ca)	1 gram of casein to 1.04 x 10^{-4} gram equivalents.

TABLE IX.— COMPOSITION OF DI-CALCIUM CASEINATE PREPARATION.

Amount of compound used.	Amount of CaO found.	Percentage of CaO in compound.	Relation of casein to calcium in compound.
Grams.	*Grams.*		
4.2825	0.0233 (0.0167 Ca)	0.544 (0.39 Ca)	1 gram of casein to 1.95 x 10^{-4} gram equivalents.
4.1215	0.0235 (0.0168 Ca)	0.572 (0.41 Ca)	1 gram of casein to 2.04 x 10^{-4} gram equivalents.
Ave. 4.202	0.0234 (0.01675 Ca)	0.558 (0.40 Ca)	1 gram of casein to 2.00 x 10^{-4} gram equivalents.

If we compare the results given in Tables VIII and IX with the figures given in paragraphs (1), (2), (3) and (4) on page 17, it is obvious that the results embodied in these tables are lower. The higher results are obtained by the volumetric method and are believed

to be nearer the truth, owing to the difficulty of preparing these caseinates in pure form. The values by the volumetric method are: 1 gram of casein to 1.10 (to 1.15) x 10^{-4} gram equivalents of calcium for the mono-basic caseinate, and 1 gram of casein to 2.2 (to 2.3) x 10^{-4} gram equivalents of calcium for the di-basic caseinate.

VALENCY OF CASEIN MOLECULE AND MOLECULAR WEIGHT OF CASEIN.

In the case of the compound of casein and calcium, which is neutral to phenolphthaleïn, it is found that 1 gram of casein combines with 9 x 10^{-4} gram equivalents of calcium. In the case of the mono-ammonium caseinate, the combination is in the proportion of 1 gram of casein to a value between 1.1 x 10^{-4} and 1.15 x 10^{-4} gram equivalents. Since we have one compound of known composition and another of approximately known composition, it should be possible by applying the rule of constant proportions to determine the true composition of the mono-basic caseinate and also the number of valencies satisfied in the caseinate neutral to phenolphthaleïn.

We have reason to believe that the proportion, 1 gram of casein to 1.125 x 10^{-4} gram equivalents of alkali, is the true value, since, first, this lies between the two limits (1.10 and 1.15) found in our volumetric work, and, second, this figure agrees with that found by assuming a valency of 8 for the basic calcium caseinate, in which 1 gram of casein combines with 9 x 10^{-4} gram equivalents of calcium. Thus, if the valencies satisfied are 8, the proportion becomes 1 gram of casein to 1.125 x 10^{-4} gram equivalents of alkali for mono-basic caseinates. If, however, we were to assume that the number of valencies in the basic compound is 7 rather than 8, then the mono-basic salt would, theoretically, have the composition, 1 gram of casein to 1.285 x 10^{-4} gram equivalents of alkali, a value too high for our analytical results. If, on the other hand, we were to assume the number of valencies in the basic compound to be 9, then the proportion in the mono-basic compound would become 1 gram of casein to 1 x 10^{-4} gram equivalents of alkali, a value too low for our analytical results obtained with mono-ammonium and other alkali caseinates. Assuming 8 as the true valency of basic calcium caseinate gives us the value, 1 gram of casein to 1.125 x 10^{-4} gram equivalents of alkali, a result which agrees with the volumetric results obtained in case of the mono-alkali caseinates.

If we use the sulphur content as a basis for calculating the molecular weight of casein we have $n(\frac{32}{0.72})\ 100 = n4454+$. Here the value of n appears to be 2, and the molecular weight would be 8908, which is in very close agreement with the value found above, 8888+. This would indicate that there are two atoms of sulphur in each molecule of casein.

The amount of phosphorus in casein was found to be 0.71 per ct., which would lead to the molecular weight, $n(\frac{31}{0.71})\ 100 = n4372—$. If the value of n is 2, the molecular weight of casein becomes 8744.

On the basis of 8 representing the true number of valencies satisfied in the basic-calcium caseinate molecule, the molecular weight of casein is $\frac{1}{1.125 \times 10^{-4}}$ or 8888+. Robertson[1] reaches similar results by deducing the molecular weight of casein in several different ways. This would also make the equivalent weight of casein equal to $\frac{8888}{8}$ or 1111. This value is in close agreement with the equivalent weight assigned by other workers to casein prepared from cow's milk. Laqueur and Sackur[2] give about 1135; Matthaiopoulos[3] gives 1131.5; Long[4] gives 1124.

As a result of the work here reported, it would seem possible, theoretically, to prepare a series of not less than eight combinations of casein with each of the basic elements studied.

According to what we have reason to believe at the present time, not less than four of these combinations have been prepared. Using the calcium compounds, we have the following series:

Name of compound.	Grams Ca for 100 grams of casein.	Valencies satisfied.
Mono-calcium caseinate	0.22 (equal to 0.31 CaO)	1
Di-calcium caseinate	0.44 (equal to 0.62 CaO)	2
Neutral calcium caseinate	1.07 (equal to 1.50 CaO)	5
Basic calcium caseinate	1.78 (equal to 2.50 CaO)	8

It is noticeable that in this series compounds are absent representing valencies of 3, 4, 6 and 7. Whether such compounds can be prepared no one can say at present.

PART II. PARACASEIN AND SOME OF ITS COMPOUNDS.

The term paracasein is generally applied to the precipitated protein compounds formed by treating milk with rennet-extract. The relations between casein and paracasein are not satisfactorily understood as yet. Little study has been given to the compounds formed by paracasein. Van Slyke and Hart[5] have shown that paracasein combines with calcium to form a compound neutral to phenolphthaleïn, containing about 2.40 per ct. CaO (1.71 per ct. Ca), or 1 gram of paracasein combines with 8.55×10^{-4} gram equivalents of calcium. They also found a compound neutral to litmus, in which there was about 1.50 per ct. CaO (1.07 per ct. Ca), or 1 gram of paracasein combines with 5.35×10^{-4} gram equivalents of calcium. According to their results, the compound containing the

[1] *Jour. Physical Chem.*, **15**: 179, 1911.
[2] *Hofmeister's Beiträge*, **3**: 193, 1902.
[3] *Ztsch. Analyt. Chem.*, 47: 492, 1908.
[4] *Jour. Am. Chem. Soc.*, **28**: 372, 1906.
[5] N. Y. Agr. Exp. Sta. Bull. No. 261, and *Am. Chem. Jour.*, **33**: 472, 1905.

higher amount of calcium is soluble in water, while the other is insoluble. It will be later shown by us that both of these compounds are soluble in pure water. In reporting the compound neutral to litmus to be insoluble in water, the fact was overlooked that, under the conditions of their experiments, there was always present in the mixture a considerable amount of calcium chloride, which was formed by the reaction of the hydrochloric acid upon the lime-water solution of paracasein, and the presence of this calcium chloride caused the precipitation of the neutral calcium paracaseinate, as we shall show later.

We have been able to prepare compounds of paracasein corresponding to the mono- and di-basic caseinates, in which, however, the proportion by weight of paracasein to base is just one-half that found in the caseinates.

PREPARATION AND COMPOSITION OF ASH-FREE PARACASEIN.

Milk from which the fat has been removed as completely as possible by centrifugal force was heated to 37° C. and rennet-extract (Hansen's) was added in the proportion of 0.12 cc. per 1,000 cc. of milk. The milk was allowed to stand until the precipitated paracaseinate had separated as completely as possible. The resulting curd was then stirred vigorously in order to break it into small pieces and hasten the separation of the whey. When the curd had settled, the supernatant whey was removed by siphon. The paracaseinate was washed with distilled water several times, and finally 5 liters of water were added for each liter of milk originally used. Dilute ammonium hydroxide (6 cc. of strong reagent diluted to 1,000 cc.) was then added, as in the case of the preparation of casein (p. 8), and the mixture stirred until the paracaseinate was dissolved. The process of precipitating, washing and redissolving was continued as in the case of casein; the remaining calcium was finally separated by addition of ammonium oxalate and centrifuging. One preparation made in this way contained 0.07 per ct. of ash. One gram gave a clear solution when dissolved in 10 cc. of $\frac{N}{10}$ NH_4OH and 90 cc. of water. One preparation, with high ash content, gave the following results on analysis:

	Per ct.	In dry substance:	*Per ct.*
Moisture	1.63	Nitrogen	15.80
Ash	0.61	Phosphorus	0.83
In dry substance:		Sulphur	0.87
Carbon	53.50	Oxygen (by difference)	21.13
Hydrogen	7.26		

Another preparation with exceptionally low ash content gave the following results:

Ash..... 0.07 per ct. Phosphorus.... 0.71 per ct. Sulphur..... 0.72 per ct.

PREPARATION AND COMPOSITION OF BASIC CALCIUM PARACASEINATE.

Like casein, paracasein manifests its acid character by its power to liberate carbon dioxide from calcium carbonate, forming a calcium paracaseinate. The results of the reaction were measured by us in the same manner as in the case of casein (p. 10), and it is, therefore, not necessary to report any of the details of methods or results. The average of many determinations indicates that paracasein unites with calcium to form a paracaseinate which is neutral to phenolphthaleïn and has the same general composition; 1 gram of paracasein combines with 9 x 10^{-4} gram equivalents of calcium.

PREPARATION AND COMPOSITION OF UNSATURATED OR ACID PARACASEINATES.

In preparing acid paracaseinates of bases, the same volumetric method of procedure was followed as in case of the casein salts (p. 12). The appearance of a precipitate in a centrifuged portion after addition of acid to an alkali solution of paracaseinate was made to serve as an indicator in regard to the end point of the reaction. We dissolved 5 grams of the ash-free paracaseinate in 200 cc. of $\frac{N}{40}$ alkali, made up the solution to 250 cc. and then determined the end points by careful addition of $\frac{N}{50}$ HCl to 50 cc. portions.

Acid paracaseinates of ammonium, sodium and potassium.— In the manner described, determinations were made in the case of base-free paracasein dissolved in hydroxide of ammonium, sodium and potassium, with the results tabulated below:

TABLE X.—RELATION OF ALKALI BASES TO PARACASEIN IN ACID PARACASEINATES.

Amount of paracasein used.	Kind of alkali used.	Amount of $\frac{N}{50}$ alkali used.	Amount of $\frac{N}{50}$ HCl required to cause first sign of permanent precipitate.	Amount of alkali left combined with paracasein.		Amount of $\frac{N}{50}$ HCl required to precipitate all of paracasein.
				$\frac{N}{50}$	$\frac{N}{10}$	
Gram.		*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*
1	NH_4 OH	50	Between 38.5 and 39	11 to 11.5	2.2 to 2.3	50
1	Na OH	50	" " " "	" "	" "	"
1	KOH	50	" " " "	" "	" "	"

These results show that 1 gram of paracasein combines with an amount of alkali somewhere between 2.2 x 10^{-4} and 2.3 x 10^{-4} gram equivalents, in forming soluble compounds with ammonium, sodium and potassium, which are acid to both litmus and phenolphthaleïn. Expressed in another form, 1 cc. of $\frac{N}{10}$ alkali combines with an amount of paracasein somewhere between 0.435 and 0.455 gram. The proportion of basic element in each compound is approximately as follows: NH_4, 0.40 per ct.; Na, 0.52 per ct.; K, 0.88 per ct. The amount of each basic element in these paracaseinates is just double that present in the corresponding casein compounds (p. 14).

The amount of acid required to precipitate completely the paracasein in these compounds is exactly equal to the alkali used to dissolve the paracasein; this indicates that there is no additional paracaseinate, in insoluble form, containing less of these basic elements.

Preparation of mono-ammonium paracaseinate.— This compound was isolated and prepared in dry form, for futher study, in the manner already described in the preparation of mono-ammonium caseinate (p. 14). Care must be taken to use a paracasein preparation free from casein or salts of calcium, strontium, barium, etc. A determination of the amount of ammonia present in preparations thus made is given below.

Table XI.— Composition of Mono-Ammonium Paracaseinate.

Amount of paracaseinate used.	Amount of $\frac{N}{10}$ NH_4OH found.	Relation of paracasein to NH_4OH in paracaseinate.	Percentage of NH_4 in paracaseinate.
Grams.	*Cc.*		
4	8.20	1 gram of paracasein to 2.05×10^{-4} gram equivalents	0.37
4	7.98	1 " " " 2.00×10^{-4} " "	0.36

These results show the difficulty of making a pure compound, but they indicate that the percentage of ammonium is double that found in the corresponding mono-ammonium caseinate.

Acid paracaseinates of calcium, strontium and barium.— In preparing paracasein salts of calcium, strontium and barium, the presence of their chlorides causes much more trouble in respect to precipitation than in case of the casein salts. Special care must be taken to prevent the accumulation of chlorides of these elements. By sufficiently frequent dialysis it was possible to obtain the results reported below (Table XIII). Another point in connection with paracasein is the fact of its slow rate of solution in the hydroxides of calcium, strontium and barium; on this account we used 400 cc. of $\frac{N}{40}$ hydroxide to dissolve 5 grams of paracasein, making the volume up to 500 cc. with water.

Trial or preliminary determinations were made in the same manner as with casein (p. 15), in order to determine the amount of $\frac{N}{50}$ HCl required to precipitate the paracasein in the absence of chlorides of calcium, strontium and barium. The specific details employed and results obtained are indicated in the following table:

TABLE XII.— ILLUSTRATION OF METHOD OF PREPARING ACID PARACASEINATES OF CALCIUM, STRONTIUM AND BARIUM.

Amount of paracasein in solution.	Amount of $\frac{N}{50}$ hydroxide solution used.	Amount of $\frac{N}{50}$ HCl added.	Precipitation.	
Grams.	*Cc.*	*Cc.*		
1	100	80	Precip.	First trial.
1	100	75	0	Dialyzed and used for next.
1	100	80	Precip.	
1	100	75	0	Dialyzed and used for next.
1	100	76	0	" " " " "
1	100	77	Precip.	
1	100	75	0	Dialyzed and used for next.
1	100	76	0	" " " " "
1	100	76.5	0	" " " " "
1	100	77	Precip.	
1	100	75	0	Dialyzed and used for next.
1	100	76	0	" " " " "
1	100	76.5	0	" " " " "
1	100	77	0	" " " " "
1	100	77.5	Precip.	
1	100	75	0	Dialyzed and used for next.
1	100	76	0	" " " " "
1	100	76.5	0	" " " " "
1	100	77	0	" " " " "
1	100	77.25	0	" " " " "
1	100	77.50	Precip.	

The results obtained by the method of procedure indicated above are given in the following table for calcium, strontium and barium.

TABLE XIII.— PARACASEINATES OF CALCIUM, STRONTIUM AND BARIUM.

Amount of paracasein.	Kind of hydroxide.	Amount of $\frac{N}{50}$ hydroxide used.	Amount of $\frac{N}{50}$ HCl required to cause first sign of permanent precipitate.	Amount of base left combined with paracasein in solution.		Amount of $\frac{N}{50}$ HCl required to precipitate all paracasein.	Amount of base in precipitated paracasein.	
				$\frac{N}{50}$	$\frac{N}{10}$		$\frac{N}{50}$	$\frac{N}{10}$
Gram.		*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Cc.*
1	$Ca(OH)_2$	100	77.25 to 77.5	22.5 to 22.75	4.5 to 4.55	88.5	11.5	2.3
1	$Sr(OH)_2$	100	77.25 to 77.5	22.5 to 22.75	4.5 to 4.55	88.5	11.5	2.3
1	$Ba(OH)_2$	100	77.25 to 77.5	22.5 to 22.75	4.5 to 4.55	88.5	11.5	2.3

These results indicate the formation of two sets of compounds when paracasein is dissolved in a hydroxide of calcium, strontium or barium and this solution is neutralized with acid under the conditions of our experiments. One set of compounds contains twice as much base as the other, corresponding to the two sets of casein compounds. Additional details are discussed below:

(1) In the di-basic compounds, the results show that 1 gram of paracasein requires between 4.5×10^{-4} and 4.55×10^{-4} gram equivalents of hydroxide of calcium, strontium or barium to form a compound which is soluble in pure water. These compounds are easily precipitated from their water solutions by a minute amount of a soluble salt of calcium, strontium or barium.

(2) In these di-basic compounds, 100 grams of paracasein combine, approximately, (a) with 0.90 gram Ca (equal to 1.26 grams CaO), (b) with 1.97 grams Sr (equal to 2.33 grams SrO), or (c) with 3.09 grams Ba (equal to 3.45 grams BaO).

(3) It is indicated that, with the treatment described above, 1 gram of paracasein combines with about 2.3×10^{-4} gram equivalents of the hydroxide of calcium, strontium or barium to form an *insoluble* compound. These compounds are regarded as mono-basic paracaseinates.

(4) In these insoluble mono-basic paracaseinates, 100 grams of paracasein combine, approximately, (a) with 0.46 gram Ca (equal to 0.64 gram CaO), (b) with 1.01 grams Sr (equal to 1.19 grams SrO), or (c) with 1.58 grams Ba (equal to 1.76 grams BaO).

(5) Mono-basic paracaseinates of calcium, strontium and barium are completely soluble in warm 5 per ct. solution of ammonium, sodium or potassium chloride. This solubility is due to interchange of bases, just as in the case of caseinates (p. 18); the reaction was studied experimentally with paracaseinates and the same results obtained as in case of the caseinates.

(6) A comparison of the composition of the caseinates and paracaseinates shows that twice as much base is present in paracaseinates as in the corresponding caseinates. This is easily seen in the following table.

TABLE XIV.— COMPARISON OF COMPOSITION OF CASEINATES AND PARACASEINATES.

Amount of basic element combined with 100 *grams of casein and paracasein.*

Basic element.	In mono-basic caseinates.	In mono-basic paracaseinates.	In di-basic caseinates.	In di-basic paracaseinates.
	Gram.	*Grams.*	*Grams.*	*Grams.*
Ca.....	0.22	0.46	0.44 to 0.46	0.90
Sr.....	0.48	1.01	0.96 to 1.01	1.97
Ba.....	0.76	1.58	1.51 to 1.58	3.09

Preparation of mono- and di-calcium paracaseinates.— In order to study the composition and properties of these compounds further, preparations of the mono- and di-calcium paracaseinates were made. The first steps in making these compounds are the same. An excess of ash-free paracasein is agitated with lime-water until a saturated solution is formed, the undissolved paracasein being removed by filtration. To the solution $\frac{N}{20}$ HCl is added until a permanent precipitate begins to appear. The solution is again filtered and then dialyzed. Alternate addition of acid and dialysis are continued until no more acid can be added after dialysis without causing precipitation. The amount of $\frac{N}{50}$ HCl required to precipitate all the paracasein is next determined in an aliquot portion, and one-third that amount of acid is added. The solution is then filtered and dialyzed. This solution contains di-calcium paracaseinate. This solution is divided into two portions; in one the di-calcium paracaseinate is precipitated by addition of acid-free alcohol, the precipitate being washed with acid-free alcohol and ether and dried at 120° C. This preparation was found to contain between 4.2×10^{-4} and 4.6×10^{-4} gram equivalents of calcium for 1 gram of paracasein.

In the second portion of di-calcium paracaseinate solution, enough $\frac{N}{50}$ HCl is very slowly added to precipitate three-fourths of the paracasein in solution. The precipitate is mono-calcium paracaseinate; this is filtered, washed with acid-free alcohol and ether, and dried at 120° C. Before being washed with alcohol, the precipitate is completely soluble in 5 per ct. solution of sodium chloride. This compound, mono-calcium paracaseinate, is identical in its properties with the brine-soluble compound formed in cheddar cheese, to which attention was first called by Van Slyke and Hart under the expression, "salt-soluble compound." Attention will again be called to this compound later. An analysis of this preparation showed it to contain between 2×10^{-4} and 2.3×10^{-4} gram equivalents of calcium for 1 gram of paracasein.

VALENCY OF PARACASEIN MOLECULE AND MOLECULAR WEIGHT OF PARACASEIN.

In the case of basic calcium paracaseinate, the compound that is neutral to phenolphthaleïn, it is found that 1 gram combines with 9×10^{-4} gram equivalents of calcium, while in the case of mono-ammonium paracaseinate the combination is in the ratio of 1 gram of paracasein to a value between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents. According to the rule of constant proportions, the number of valencies satisfied in the first compound would be between $\frac{9}{2.2}$ and $\frac{9}{2.3}$ or 4. The molecular weight of paracasein would therefore be $\frac{1}{\frac{2.25}{1} \times 10^{-4}}$ or 4444+. Our results indicate that the molecular weight of casein, 8888, is just twice that of paracasein 4444. Using the sulphur content as a basis for calculating the molecular weight of paracasein, we have $n(\frac{32.07}{0.72})\ 100 = n\ 4454+$.

The percentage of phosphorus would give $n(\frac{31.04}{0.71})\ 100 = n\ 4372—$. The value of n appears to be 1 and each molecule of paracasein would contain one atom each of sulphur and phosphorus.

Theoretically, it should be possible to make a series of four salts of paracasein with bases. We have prepared three, those in which one, two and four valencies are satisfied.

ACTION OF RENNET ENZYM ON CASEIN IN FORMING PARACASEIN.

The action of the principal enzym contained in rennet-extract in splitting casein into two molecules of paracasein is further shown by the following experiment: Five grams of casein are dissolved in 250 cc. of $\frac{N}{50}$ KOH. Using the volumetric method given on page 13, it was found that 44.5 cc. of $\frac{N}{50}$ HCl could be added to 50 cc. of the caseinate solution, containing 1 gram of casein, before a permanent precipitate begins to appear. To another 50 cc. of caseinate solution a few drops of neutral rennet-extract are added. Under the conditions of the experiment, no precipitate or curd is produced by the action of the rennet-enzym. After a few minutes $\frac{N}{50}$ HCl is added and it is found that a permanent precipitate begins to form as soon as we add only 39 cc. $\frac{N}{50}$ HCl.

We have in hand a more extended investigation relating to the action of rennet-enzym upon casein, the results of which will be published later.

PART III. COMPOSITION OF THE BRINE-SOLUBLE COMPOUND IN CHEESE.

During the manufacture and ripening of cheddar cheese and of many other kinds of cheese there is always found a protein that is soluble in a warm 5 per ct. solution of sodium chloride. The existence of such a substance in cheddar cheese was first brought to attention by work done at this Station.[1] The presence of this brine-soluble protein was shown to be associated in some way with the formation of acid in the cheese and, on the basis of some early experiments, Van Slyke and Hart were led to conclude erroneously that the substance consists of a combination of paracasein and lactic acid (called by them paracasein mono-lactate), which by the addition of more lactic acid becomes insoluble in dilute brine solution, forming a compound which they mistakenly regarded as paracasein di-lactate. As a result of later work[2] they changed their first views and came to the conclusion that the so-called paracasein mono-lactate is simply the uncombined protein, paracasein, and that the so-called paracasein di-lactate is a compound of paracasein and

[1] N. Y. Agr. Exp. Sta. Bul. No. 214, 1902.
[2] N. Y. Agr. Exp. Sta. Bul. No. 261, 1905.

lactic acid (1 gram of paracasein uniting supposedly with about 0.5 cc. $\frac{N}{10}$ acid). It may be stated here, in passing, that it was later shown by L. L. Van Slyke and D. D. Van Slyke[1] that the protein casein does not unite with acids to form insoluble compounds, but that the action is simply one of *adsorption*, by which more or less acid is taken from the surrounding solution and concentrated on the surface of the solid particles of protein; in other words, it was shown that casein or paracasein mono-lactate or di-lactate have no existence as applied to the compounds in question. It still remained, therefore, to find out what the brine-soluble substance really is, and work was continued along this line by the writers.[2] We noticed that calcium is always to be found associated with the brine-soluble substance when it is separated from the other cheese constituents by extraction with a solution of calcium-free sodium chloride after previous removal of all water-soluble constituents. This fact suggested the possibility that the brine-soluble substance might be a combination of paracasein and calcium, containing less calcium than had been previously found in any combination of this element with paracasein. On the basis of such a possibility, it could be explained that with the formation of increased amounts of lactic acid in cheese-making, as a result of the bacterial decomposition of milk-sugar, the acid would combine with more or less of the calcium contained in calcium paracaseinate, resulting in the production of a para-caseinate containing less calcium. This suggestion was strengthened by the fact that in Camembert cheese, the brine-soluble compound is formed during certain stages of the manufacturing process but soon disappears, its formation and disappearance being explained as follows according to Bosworth:[3] The brine-soluble substance is at first formed in Camembert cheese, as also in the case of cheddar cheese, but, owing to the method of making this type of cheese, more acid is allowed to form in the cheese, and, as a consequence, the brine-soluble substance loses its calcium and becomes free para-casein, which is insoluble in brine solution. Therefore, in the manufacture of Camembert cheese, it is found after the first few hours that the cheese contains no brine-soluble material and, what is also significant, all the calcium is found in the water extract. The relation between the brine-soluble substance and the calcium found in the brine extract in the two types of cheese is illustrated in Table XV.

The question necessarily suggests itself as to whether the calcium always found in the brine-soluble extract of cheese is not there incidentally in a mechanical state rather than in combination with paracasein. In order to study this question the following work

[1] N. Y. Agr. Exp. Sta. Tech. Bul. No. 3, 1906.
[2] N. Y. Agr. Exp. Sta. Tech. Bul. No. 4, 1907.
[3] N. Y. Agr. Exp. Sta. Tech. Bul. No. 5, 1907.

Table XV.— Comparison of Changes in Cheddar and Camembert Cheese.

Age of cheese.	Kind of cheese.	Total nitrogen in the form of brine-soluble compound.	Total calcium found in brine-soluble compound.
		Per ct.	*Per ct.*
When curd was cut	Cheddar	3.13	trace.
	Camembert	6.72	trace.
10 hours	Cheddar	96.00	27.96
	Camembert	94.00	17.76
2 days	Cheddar	68.87	24.47
	Camembert	4.39	trace.
4 months	Cheddar	43.09	24.28

was done: Twenty-five grams of cheese were ground with sand and extracted with water at about 55° C., using 150 cc. portions, until the extract amounted to 1,000 cc. The residue, containing the brine-soluble substance, was placed in a dialyzing apparatus and allowed to dialyze to insure the removal of all soluble calcium. Sodium chloride was then added to the contents of the dialyzing tube, which was then placed in a beaker of water and allowed to remain 4 hours. Upon adding ammonium oxalate to some of the water in the beaker, a precipitate of calcium oxalate appeared. This result leads to the belief that the calcium is present in combination in an insoluble form and that an interchange takes place between it and sodium, when the insoluble compound is treated with sodium chloride solution.

In order to throw further light on the character of the brine-soluble compound, a study was made of the solvent effect of several different chlorides. One kilogram of cheddar cheese was ground fine, thoroughly mixed, and then 25-gram portions were ground with sand, placed in bottles and extracted with water in the manner described in the preceding paragraph. The residues were then extracted with solutions of chlorides and the results given in the following table were obtained. The solutions of the salts were used in such strength that 1,000 cc. contained equivalent gram molecules. In the case of the weakest solution, extraction was continued as long as appreciable amounts of protein were obtained in the extract, 4,000 cc. being used; the results in these cases are given for each 1,000 cc. of extract, as well as for the total.

In connection with the data in Table XVI, attention is called to certain phases of the results.

(1) The chlorides of barium and calcium have no solvent effect. The chloride of magnesium in strong molecular concentrations acts much like the chlorides of sodium, ammonium and potassium, while in lower molecular concentrations its solvent power is greatly reduced.

TABLE XVI.— SOLVENT ACTION OF NEUTRAL CHLORIDES ON THE BRINE-SOLUBLE COMPOUND IN CHEESE.

Strength of solution. Gram equivalents per 1,000.	Amount of extract.	Percentage of total nitrogen in water-insoluble residue of cheese extracted by —					
		Na Cl.	NH_4 Cl.	KCl.	$MgCl_2$	$BaCl_2$	$CaCl_2$
	Cc.						
1.0.....	1,000	68.57	67.62	50.47	63.81	0	0
0.8.....	1,000	69.29	65.24	50.47	48.33	0	0
0.6.....	1,000	56.19	56.43	45.95	lost	0	0
0.4.....	1,000	51.43	51.19	44.52	23.57	0	0
0.2.....	1st 1,000	47.62	49.05	40.95	4.00	0	0
0.2.....	2nd 1,000	13.33	10.48	13.90	5.24	0	0
0.2.....	3rd 1,000	2.95	4.10	2.00	4.29		
0.2.....	4th 1,000	trace	trace	trace	—		
Total.....	4,000	63.90	63.63	56.85	—		

(2) Sammis and Hart[1] undertook to study the solvent effect of these salts on the same material, but reached results not concordant with one another and not in agreement with ours. While we used solutions of such strength as to show the relation existing between the solvent action of the salt solution and its molecular concentration, they used solutions containing a uniform percentage by weight of different salts and extracted in every case with the same volume of solution. By using solutions of different salts having the same percentage composition by weight, but with a different molecular concentration, one would, under the circumstances, expect to obtain only discordant results, because the solvent effect of the solution is apparently a result of the mass action of the salt in solution (p. 18). If Sammis and Hart had in their work continued extraction until no more solvent effect was appreciable, their results would have been in satisfactory agreement with ours. This is strikingly shown in the above table in the case of the 0.2 normal solutions; by continued extraction, the total amounts extracted are found to be essentially the same as in case of the more concentrated solutions.

IDENTITY OF THE BRINE-SOLUBLE COMPOUND OF CHEESE WITH MONO-CALCIUM PARACASEINATE.

We have shown (p. 26) that paracasein combines with calcium to form a compound insoluble in water but soluble in 5 per ct. solution of sodium chloride (sodium replacing calcium). In this compound, we have shown, 1 gram of paracasein is in combination with

[1] *Jour. Biol. Chem.*, 6: 181, 1909.

2.25×10^{-4} gram equivalents of calcium. Indications pointed to the identity of the brine-soluble compound of cheese with this mono-calcium paracaseinate, and it remained to ascertain whether the protein part of the molecule in these two compounds is the same. In order to accomplish this, a preparation of the protein in the brine-soluble compound was made from cheese and its composition and properties were studied.

One kilogram of cheddar cheese was ground fine and then extracted with numerous portions of distilled water at about 55° C. in order to remove all soluble compounds. The residue was then extracted with many portions of a 5 per ct. solution of sodium chloride and filtered, first through absorbent cotton and then through paper. Dilute acetic acid was then added, giving a heavy precipitate, which was washed with water, redissolved in dilute ammonia and again precipitated with acid. The process was then completed as in the preparation of casein (p. 9). The preparation on analysis gave the following results, which are probably high, owing to the ash content:

	Per ct.		*Per ct.*
Moisture	2.32	In dry substance:	
Ash	0.25	Nitrogen	15.82
In dry substance:		Phosphorus	0.75
Carbon	52.97	Sulphur	0.78
Hydrogen	7.15	Oxygen (by difference)	22.28

A study of the properties of this substance resulted as follows:

(1) The substance acts as an acid in combining with bases.

(2) It decomposes calcium carbonate and gives a compound in which 100 grams of substance combines with the equivalent of 2.52 grams CaO (equal to 1.80 grams Ca), or 1 gram of substance combines with 9×10^{-4} gram equivalents of calcium.

(3) The solution of this calcium compound is neutral to phenolphthaleïn.

(4) Measured by the volumetric method, it was found to form a compound with ammonia represented by the combination of 1 gram of substance with 2.3×10^{-4} gram equivalents.

(5) With calcium it forms a compound soluble in 5 per ct. solution of sodium chloride but insoluble in water, which contains 1 gram of substance combined with 2.3×10^{-4} gram equivalents of calcium.

(6) It forms also a compound with calcium that is soluble in water, containing 1 gram of substance combined with 4.5×10^{-4} gram equivalents.

In view of the marked agreement of the composition and properties of the brine-soluble substance, formed in cheese, with the compound, mono-calcium paracaseinate, as prepared by us, there is good reason to believe that the brine-soluble substance is mono-calcium paracaseinate, having the composition of 1 gram of paracasein combined with 2.25×10^{-4} gram equivalents of calcium.

TECHNICAL BULLETIN No. 27. MARCH, 1913.

New York Agricultural Experiment Station.

GENEVA, N. Y.

A STUDY OF THE UDDER FLORA OF COWS.

H. A. HARDING AND J. K. WILSON.

PUBLISHED BY THE DEPARTMENT OF AGRICULTURE.

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TECHNICAL BULLETIN No. 27.

A STUDY OF THE UDDER FLORA OF COWS.

H. A. HARDING AND J. K. WILSON.

SUMMARY.

(1) An examination of 1230 samples of milk direct from the udders of 78 cows indicates that the germ content is smaller than has previously been assumed, the average of these samples being 428 per cc.

(2) The bacteria were not evenly distributed among the quarters of the udder, there being about three times as many per cc. in the back as in the front quarters. The factor controlling this distribution has not been found.

(3) Neither the age of the cow nor the period of lactation exerted any marked influence upon the germ content of the udder.

(4) When classified in accordance with the system of the Society of American Bacteriologists the organisms found in about 900 samples fell into 71 groups. The udder flora is characterized by a lack of motility, of spore formation and of gas formation. It is largely composed of micrococci and is practically all Gram positive.

INTRODUCTION.

Bacteria exist in varying numbers in the udders of all cows. Therefore even extreme cleanliness in the dairy will not reduce the germ content of the milk below the number which it contains while it is yet within the udder. The finding by Hastings and Hoffmann[1] of an apparently healthy cow with an average udder content of 191,000 germs per cc., while an extreme case, shows the possible magnitude of this phase of the milk question. With

[1] Hastings, E. G., and Hoffmann, C. Bacterial content of the milk of individual animals. Wis. Agr. Exp. Sta. Research Bul. 6. 1909. Also in Ann. Rpts. of same Station 25 & 26 (1908-1909):189-196. 1910.

the increasing emphasis which is being laid upon the germ content of milk the germs in the cow's udder are becoming more and more an important factor. They are especially important in the production of "certified" milk, and the small number of germs found in the product of some "certified" dairies is made possible by the selection of cows having few germs in their udders.

The problem of producing milk with a low germ content at a moderate cost is being studied at this Station. While the presence of bacteria in the udders of healthy cows has been recognized for some time there is very little information regarding the normal flora of udders and less regarding the forces which regulate the growth of the forms which are present. A beginning has been made on this phase of the subject by examining 1,274 samples of milk representing 83 cows. A continuation of these studies along a number of lines which have as yet been hardly touched is much to be desired. The removal of each of the authors to other institutions makes necessary the publication of the results thus far obtained even though the entire field has not been covered.

ACKNOWLEDGMENTS.

The careful collection of this large number of milk samples would have been much more difficult but for the active cooperation of our colleague, Mr. G. A. Smith, and the men in his department who willingly placed all the facilities of the barn and the dairy at our disposal and actively assisted in the work.

The kindness of Mr. A. G. Lewis in assisting us to obtain udder samples from a large number of cows, many of them recently imported from the Island of Guernsey, materially broadened the investigation.

Acknowledgment should also be made of the suggestions and aid given by Dr. R. S. Breed, while he was engaged in a study of the cellular elements in the milk of many of the same cows whose udder flora was being determined.

PREVIOUS STUDIES.

Many workers have contributed to our knowledge of this subject and it will serve the present purpose to indicate merely the steps by which this knowledge has progressed.

In 1877 Lister[2] presented a paper to the Pathological Society at London which contained the following statements: "In this case, the experiment was more rigorously conducted, and here, in the majority of the glasses, at first sight, you will suppose that no change at all had occurred; and in two of them I found, at the end of six weeks, that there was no indication whatever of any organisms. I topped one of them and found the milk still perfectly fluid, of normal taste and reaction and without any organisms in it; showing that unboiled milk, as coming from the healthy cow, really has no ferment in it capable of leading to lactic fermentation or any other fermentation, or to any organic development whatever." Thus on the basis mainly of two small samples, only one of which was critically examined, there was founded the theory that the milk within the healthy udder is germ free. This theory was generally held until about 1890.

Apparently the first quantitative study of bacteria in the udder was made under the direction of Dr. Lehmann. One of his students, Schulz,[3] found that the first milk contained large numbers of bacteria while samples drawn midway and at the close of the milking process contained progressively smaller numbers of germs. The results of this study were also presented by Lehmann (17th Versammlung d. deut. Ver. f. offentl. Gesundheitspflege, Leipsic, Sept. 1891), as the work of Schulz, after they had been published as an Inaugural Dissertation by Schulz in July, 1891. These two presentations have been confused in literature and have been frequently referred to as two separate studies.

[2] Lister, J. Lactic fermentation and its bearing on pathology. *Pharm. Jour. and Trans.* (Pathological Society of London) III Series, **8**:555–558; 572–575. 1878.

[3] Schulz, L. Ueber den Schmutzgehalt der Würzburger Marktmilch und die Herkunft der Milchbakterien. *Arch. Hyg.* **14**:260–271. 1892.

The work of Schulz was confirmed by Moore,[4] who concluded that "Freshly drawn fore milk contains a variable number of bacteria, varying in number from a few individuals to many thousand per cubic centimeter. These are distributed among several species. The last milk drawn at a regular milking contains, as compared with the fore milk, very few micro-organisms. It is the exception, however, to find a sample of milk that is free from micro-organisms unless it is taken during the latter part of the milking process from a single quarter of the udder."

As the result of these and similar observations it was held that bacteria might find their way into the teat opening and multiply to some extent in the milk cistern, but that they were washed out more or less completely during the milking process.

In his earlier paper Moore suggested the desirability of direct examinations of excised tissue from functioning udders of freshly slaughtered milch cows as a means of settling the question of the bacterial invasion of the upper portions of udders. He later[5] reported the results of such examinations and this work was extended by one of his students, Ward.[6] These direct examinations showed that bacteria were uniformly present in the finer subdivisions of the udder. The work of Bolley[7] had indicated that certain species were able to persist in an udder for considerable periods and Moore and Ward[8] studied an instance where this udder contamination was of considerable economic importance.

Later work has confirmed the conclusion of Moore and Ward that bacteria are practically always present in the udders of healthy cows but it has also shown that the numbers of bacteria

[4] Moore, V. A. Preliminary investigations concerning the number and nature of bacteria in freshly drawn milk. U. S. Dept. Agr. An. Ind. An. Rpt. **12 & 13**:261–266. 1897.

[5] Moore, V. A. The normal bacterial invasion of the cow's udder. *Proc. Soc. Prom. Agr. Sci.* **1899**:110–113.

[6] Ward, A. R. The persistence of bacteria in the milk ducts of the cow's udder. *Jour. App. Micros.* **1**:205–209. 1898; also The invasion of the udder by bacteria. N. Y. (Cornell) Agr. Exp. Sta. Bul. 178. 1900.

[7] Bolley, H. L. Ueber die Konstanz von Bakterienarten in normaler Roh- (fore) Milch. *Cent. Bak.* **II Abt. 1**:795–799. 1895.

[8] Moore, V. A., and Ward, A. R. An inquiry concerning the source of gas and taint producing bacteria in cheese curd. N. Y. (Cornell) Agr. Exp. Sta. Bul. 158. 1899.

present vary within wide limits. This is well illustrated by the work of Hastings and Hoffmann.[9] They obtained the following results from a study of the freshly drawn milk from three cows:

Name.	Days sampled.	Average per cc.	Maximum per cc.	Minimum per cc.
Brownie	61	31,000	305,000	1,700
Dorine	33	191,000	3,500,000	2,500
Merney	27	810	4,250	50

Two-thirds of these samples were taken from the pail at the close of the milking, the remainder being taken at the middle of the milking in sterile flasks. The variation in the method of sampling did not seem to affect materially the observed germ content.

It has long been held that cleanliness in the stable would influence favorably the germ content of udders but direct evidence on this point has only recently been available. Atwood and Giddings[10] tested the influence both of sealing the opening of the teat so as to exclude all external contamination and of injecting various substances into the milk cistern. Where the end of the teat was treated with carbolized vaseline and covered with adhesive tape the germ content was but little reduced. Where the cistern was treated with chemicals there was first a reduction of germ content but this was followed by a marked rise and accompanied by an inflammation of the gland.

PRESENT STUDIES.

These studies of the normal flora of cows' udders have extended over a considerable period. During 1900–02 examinations were made of the strippings from 17 cows. At each examination samples were taken from each quarter of the udder and 324 samples were studied. These examinations were made, for most part,

[9] See footnote 1.

[10] Atwood, H., and Giddings, N. J. Experiments in the production of sanitary milk. W. Va. Agr. Exp. Sta. Bul. 134. 1911.

either by L. A. Rogers or J. F. Nicholson.* The samples were collected in sterile test tubes, at times with most elaborate preparation of the animal, at others with no preparation aside from the ordinary grooming. The results did not indicate that these variations in the preparation of the animal affected the germ content of the samples.

Plates were made promptly on agar or gelatin and at times on both. The gelatin plates were ordinarily counted at the end of five days at room temperature, the agar plates after three days at 37°C. Owing to the undeveloped state of bacteriological classification at that time only quantitative results were obtained. While these observations were merely preliminary they were of some value in establishing the range of bacterial content which may be expected in practice.

During 1907–11 one of us (W) collected and studied 766 samples from 20 cows in the Station herd. Only quantitative results were obtained from approximately the first 50 of these samples but the remainder were studied both quantitatively and qualitatively. Owing to the lapse of time since the study of 1900–02, the herd had entirely changed. The destruction of the Station barns by fire in 1904 had further changed conditions so that these two sets of observations of the Station herds have the same relative value as would the study of two separate herds.

This somewhat extended study of conditions in these two herds was supplemented by a study of a sample from each quarter of the udder of 46 cows belonging to Mr. A. G. Lewis or a total of 184 samples from this herd.

Accordingly, the present publication is a discussion of the quantitative relationships of 1,274 samples representing 83 cows and of the qualitative findings from 900 samples representing 63 cows.

* Mr. Rogers is now head of the research laboratory of the Dairy Division of the Bureau of Animal Industry, U. S. Department of Agriculture, and Prof. Nicholson, head of the Bacteriological Department of the University of Idaho. When the above work was done they were Assistant Bacteriologists at this Station.

TECHNIQUE.

The last 922 samples were obtained and handled in accord with the following general outline:

All samples were collected from the afternoon milkings and those from the Station herd were taken from each cow on three successive days. The cows from which the samples were taken had been carefully groomed but no local disinfection of the udder was attempted. The milking was done by hand, in the ordinary way, directly into sterile test tubes. With the exception of a single series, where the object was to test the condition of the milk at the various stages of milking, the samples were from the close of the milking. Care was exercised that the sample was the product of a single stream of milk and that the sterile tube was opened for the minimum time and was held in such a manner as to be exposed to the minimum amount of contamination.

The samples from the Station cows were taken to the laboratory and the plating completed within an hour, those from other sources within three hours. The medium used was as follows:

Agar	15 grams.
Peptone (Witte)	10 "
Lactose, c.p.	10 "
Beef extract (Liebig)	5 "

Water (dist.) to make a volume of 1000 cc.

The reaction of this medium ordinarily was between 1.2 and 1.5 per ct. normal acid to phenolphthalein. Whenever it exceeded the higher figure it was reduced by the addition of NaOH. This medium was chosen to permit incubation both at 20° C. and 37° C.

In plating, one cubic centimeter of milk was divided between two petri dishes and approximately 8 cc. of the agar was added to each plate. The sum of the colonies developing on these two plates was taken as the germ content of the milk. The plates were held at room temperature (20–23°C.) for five days and the first count made. They were then placed at 37°C. for two additional days and counted again. All counts were made under a lens magnifying four diameters.

In order to simplify the presentation the numerical results given in this publication are those obtained after an incubation for five days at 20–23° C. followed by two days at 37° C. unless otherwise stated.

EFFECT OF INCUBATION TEMPERATURE ON THE COUNT.

The periods of incubation officially recommended are five days at 21°C. or two days at 37°C. The lower temperature is ordinarily chosen, since gelatin, which is preferred in qualitative work on account of its better differentiation of colonies, will not remain solid at the higher temperature.

The accuracy of this five-day incubation of agar plates was tested as follows: Samples were collected at the beginning, middle and close of the milking, from each quarter of the udder of five cows and plated on agar. Counts of the plates were made after incubating five days at 20–23° C. and again after two additional days at 37° C. The percentage of increase found at the second count of the 18 samples from each quarter is given in Table I.

TABLE I.— VARIATION IN BACTERIAL COUNT AFTER ADDITIONAL INCUBATION AT 37° C.

(First count made after 5 days at 20–23° C.)

NAME.	INCREASE AT SECOND COUNT.			
	R. F.	R. B.	L. F.	L. B.
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*
Hammond No. 2	102	85	585	314
Millie G	38	0	14	8
Carey F	25	3	11	3
Gertie No. 3	410	2	18	25
Hammond F. No. 2	21	9	46	10

REMARK.— Letters R. F., R. B., etc., indicate, respectively, the right front, right back, left front and left back quarter of udder.

From the above table, which gives the results from a study of 360 samples of milk, it is seen that the effect of incubation at higher temperature is most marked in the milk from Hammond No. 2 and least in that from Carey F.

With Millie G. the results of both counts were the same from the right back quarter while the count after incubation at 37°C. was the higher in 5 samples from the right front, 11 from the left front and 4 from the left back out of the 18 samples examined from each quarter.

This marked variation in the effect of high temperature is due to variations in the flora. As will be described under the head of udder flora there were isolated 11 micrococci, a bacterium and a yeast, the colonies of which did not ordinarily appear at the lower temperature. Naturally these forms were more common in the udders of particular cows and were more frequently found in some quarters than in others. The uneven distribution of these forms in the samples is to be ascribed in part to the formation of clumps or colonies within the udder as has been described by Breed.[11] These colonies are occasionally observed in samples from the udder by means of his technique and the number of colonies appearing on the plate is dependent upon the extent to which these clumps are broken up in the plating process. It is practically certain that our present plate methods give an inadequate idea of the numbers of bacteria actually present in freshly drawn cows' milk.

CHOICE OF METHODS OF SAMPLING.

In undertaking a combined quantitative and qualitative study of the udder germs it was necessary to obtain samples which were representative both of the total numbers and of the kinds of germs present. To obtain accurately the total numbers it would seem desirable to draw a sample from the well mixed product of a quarter which had been milked under careful conditions. However, under the best of conditions some contamination will occur during the milking process and this procedure would raise the question as to whether organisms isolated from such samples were really representatives of the udder flora.

[11] Breed, R. S. The determination of the number of bacteria in milk by direct microscopical examination. *Cent. Bak.* II Abt. **30**:337–340. 1911.

The possibility of taking a sample direct from the udder which would give a satisfactory measure of the bacteria present and at the same time reduce the danger of outside contamination to the minimum was tested by milking into sterile test tubes at the beginning, the middle and at the close of the milking process. These samples were obtained from five cows on six successive days and the results of the counts summarized in Table II.

TABLE II.— COMPARISON OF BACTERIAL CONTENT AT BEGINNING, MIDDLE AND END OF MILKING.

NAME.	FIRST OF MILKING.				MIDDLE OF MILKING.				LAST OF MILKING.			
	R. F.	R. B.	L. F.	L. B.	R. F.	R. B.	L. F.	L. B.	R. F.	R. B.	L. F.	L. B.
Hammond No. 2.....	112	511	422	494	103	109	89	35	12	69	95	134
Millie G.....	2,221	3,546	73	436	681	1,714	48	272	1,426	2,715	7	280
Carey F.....	8	342	8	40	15	48	4	29	4	67	13	7
Gertie No. 3.....	703	68	8	48	339	3	4	10	246	1	3	5
Hammond No. 2.....	9	8	5	92	5	5	4	74	34	24	4	334
Average.....	612	895	103	222	271	376	26	84	344	575	24	152
General average.....	458				187				274			

REMARK.— Letters R. F., R. B., etc., indicate, respectfvely, the right front, right back, left front and left back quarter of udder.

The results from these 360 samples accord with the observation of Stocking[12] in that those at the middle of the milking contain a smaller number of germs than those taken either toward the beginning or at the close of the process.

The average germ content of the entire 360 samples was 306 per cc. The averages for the three successive sets of samples were 458, 187 and 274 respectively. From this it is seen that the average number of germs in the strippings, 274, was much more nearly equal to the grand average, 306, than the average from either of the other sets of samples. The agreement between these two numbers is reasonably close.

The accuracy of the samples of strippings as a measure of the germ content of the udder was further tested by examining

[12] Stocking, W. A. Quality of milk as affected by common dairy practices. Conn. (Storrs) Agr. Exp. Sta. Ann. Rpt. **18** (1906):66–90. 1907.

samples from the strippings and from the whole milk of a cow on three successive milkings. In this experiment the cow was carefully groomed, her flanks and udder were moistened and her teats and the hands of the milker were vaselined. A half-pint of milk was rejected from each test and the remainder of the milk from each quarter was milked into a sterile four-inch funnel, placed in a sterile bottle. Plates were promptly made from a sample of the strippings and from a sample of the thoroughly mixed milk from each quarter. The results from these plates are given in Table III.

TABLE III.— RELATION OF BACTERIAL CONTENT OF ENTIRE YIELD OF MILK AND OF STRIPPINGS.

DATE.	R. F.		R. B.		L. F.		L. B.	
	Whole milk.	Strippings.	Whole milk.	Strippings.	Whole milk.	Strippings.	Whole milk.	Strippings.
6/11/P.M.	.....	56	401	716	53	26	140	173
6/12/A.M.	216	429	629	.....	646	244	442	493
6/12/P.M.	36	305	789	975	88	22	96	105
Average.	126	263	606	845	262	97	226	257

Average of strippings, 365.
Average of whole milk, 305.
REMARK: Letters R. F., R. B., etc., indicate, respectively, the right front, right back, left front and left back quarter of udder.

From these results it is seen that while there is a fairly good agreement between the germ content found in the strippings and in the whole milk the germs are distinctly more abundant in the strippings. It should be remembered that in this case one-half pint of the first milk from each quarter was rejected, thus reducing the number of germs found in the whole milk.

It will be noted from Table II that the germs in the strippings are slightly less abundant and from Table III slightly more abundant than in the corresponding whole milk. These results suggest that with a larger number of observations the germ content of the strippings would be found to be practically identical with that of the milk of each drawing considered as a whole.

In view of these results and of the desirability of obtaining samples with as little contamination as possible the following studies of the germ content of udders were made with samples drawn, at the close of the milking process, directly into sterile test tubes.

OBSERVED NUMERICAL CONTENT.

Ordinarily it is desirable to present the detailed results so that each student of the subject may form independent judgment regarding existing relations. In the present instance limitations of space and the tediousness of presenting the merely numerical results from over 1,000 samples led to the collection of the data under the following headings:

TABLE IV.— AVERAGE BACTERIAL CONTENT OF MILK IN UDDER.

	RIGHT FRONT QUARTER OF UDDER.			LEFT FRONT QUARTER OF UDDER.			RIGHT BACK QUARTER OF UDDER.			LEFT BACK QUARTER OF UDDER.		
	Total No. germs.	No. samples.	Average per cc.	Total No. germs.	No. samples.	Average per cc.	Total No. germs.	No. samples.	Average per cc.	Total No. germs.	No. samples.	Average per cc.
Herd of 1900–02.....	33,109	79	419	29,074	77	378	52,263	80	653	49,387	80	617
Herd of 1910–11.....	36,758	185	199	22,702	174	130	117,730	185	636	129,841	186	698
Herd of A. G. L.....	7,414	46	161	4,925	46	107	27,484	46	597	15,718	46	342
Averages.....	.....	....	249	.....	....	191	.....	....	635	.....	....	625

Average germ content per cc. in 316 samples from herd of 1900–02.....	518
Average germ content per cc. in 730 samples from herd of 1910–11.....	420
Average germ content per cc. in 184 samples from herd of A. G. L.....	320
Average germ content per cc. in 1230 samples from 78 cows.....	428

Since it is important to know not only the average number of germs present in the udder but also the maximum number present at any one time, the data have been arranged in Table V to show the number of samples which contained over 1,000 germs per cubic centimeter as well as the maximum number found in samples from each quarter of the udder in each of three herds.

TABLE V.— SAMPLES CONTAINING MORE THAN 1000 GERMS PER CUBIC CENTIMETER.

HERD.	R. F. QUARTER. SAMPLES.			L. F. QUARTER. SAMPLES.			R. B. QUARTER. SAMPLES.			SAMPLES.		
	Total.	Above limit.	Maximum germ content.	Total.	Above limit.	Maximum germ content.	Total.	Above limit.	Maximum germ content.	Total.	Above limit.	Maximum germ content.
	No.	*No.*	*Per cc.*	*No.*	*No.*	*Per cc.*	*No.*	*No.*	*Per cc.*	*No.*	*No.*	*Per cc.*
1900–02.....	79	10	4,256	77	9	4,632	80	16	9,967	80	13	16,000
1910–11.....	185	12	4,120	174	8	3,130	185	31	14,280	186	33	15,750
A. G. L.....	46	2	3,180	46	2	1,340	46	5	16,610	46	4	5,280
Totals....	310	24	4,256	297	19	4,632	311	52	16,610	312	50	16,000
Above limit..	8 per ct.			6 per ct.			17 per ct.			16 per ct.		

Per ct. of all samples above limit, 12.
Maximum observed germ content per cc., 16,610.

The maximum content here given, 16,610, may not be the highest actually present among the more than 1,200 samples, since there were four samples from the herd of 1900–02 and two from the herd of 1910–11 where the plates were not counted on account of the large number of colonies present. Three of the four earlier samples were from a single cow and the two later samples were obtained on the same day from another cow.

TABLE VI.— DISTRIBUTION OF HIGH-GERM-CONTENT SAMPLES IN HERD OF 1910–11.

	HERD OF 1910–1911.											
	RIGHT FRONT QUARTER OF UDDER.			LEFT FRONT QUARTER OF UDDER.			RIGHT BACK QUARTER OF UDDER.			LEFT BACK QUARTER OF UDDER.		
	Sample.	Over 1,000	Maximum.	Sample.	Over 1,000	Maximum.	Sample.	Over 1,000	Maximum.	Sample.	Over 1,000	Maximum.
Gertie F. No. 3.....	9	0	.....	9	0	.....	9	0	.....	9	0	.....
Carey S.....	3	0	.....	3	0	.....	3	0	.....	3	0	.....
Anna G.....	3	1	1,000	3	0	.....	3	0	.....	3	0	.....
Carey D. H.....	3	0	.....	3	0	.....	3	0	.....	3	0	.....
Hammond F. No. 2..	9	0	.....	9	0	.....	9	0	.....	9	1	1,730
Carey F.....	9	0	.....	9	0	.....	9	0	.....	9	0	.....
Millie G.....	10	7	2,700	10	0	.....	10	8	10,440	10	0	.....
Hammond No. 2.....	9	0	.....	9	0	.....	9	0	.....	9	0	.....
Millie D.....	26	4	4,120	26	6	3,130	26	12	14,280	26	7	3,780
Millie F. B. B.....	15	0	.....	15	0	.....	15	0	.....	15	0	.....
Mabel S. F.....	15	0	.....	15	2	1,254	15	0	.....	15	0	.....
Ruth S.....	12	0	.....	.....	.....	.....	12	3	4,340	12	6	15,750
Gertie F. No. 2.....	24	0	.....	24	0	.....	24	0	.....	24	9	10,458
Ruth F.....	23	0	.....	24	0	.....	23	8	6,720	24	10	5,040
Millie F.....	15	0	.....	15	0	.....	15	0	.....	15	0	.....
	185	12	.....	174	8	.....	185	31	.....	186	33	.....

The fact that the small number of samples containing over 1,000 per cc. were not evenly distributed over the herd but were obtained from a relatively small number of the cows is shown by Table VI.

It will be seen that of these 15 cows only three gave samples containing over 1,000 per cc. from the front right quarter, two from the front left quarter, four from the back right and five from the back left quarter.

These rather low numbers are in contrast with many of the earlier observations, particularly those of Hastings and Hoffmann.[13] These investigators were plainly working with two very unusual animals, though none the less interesting and worthy of study on this account. The high numbers frequently obtained by other investigators were usually due to samples drawn early in the milking process and influenced by the initial high numbers of the fore milk.

Another point clearly shown in Tables IV and V is the striking difference in germ content between the front and the back quarters of the udder. According to Table IV the average content of the samples from the front quarter was 191 and 249 per cc., respectively, while those from the back quarters gave averages of 625 and 635 per cc. That this is not an accidental relationship is further indicated by the fact that in each of the three herds studied the average germ content of the back quarters is from two to four times that of the corresponding front quarters.

This same relationship is brought out in Table V, where it is shown that of the samples from the front quarters of the udder only 6 and 8 per ct., respectively, had a germ content of over 1,000 per cc., while this number was exceeded in 16 and 17 per ct., respectively, of the samples from the back quarters. The maximum germ content found in samples from the front quarters was 4,632 per cc., while over 16,000 per cc. were found in the back quarters.

A comparison of the germs found in the sets of samples taken from a single cow on any given evening, in the case of the 11

[13] See footnote 1.

cows which were most frequently tested in the Station herd, shows that the germs were more numerous in the back quarters in 68 per ct. of the 213 sets of samples. The number of these sets of samples varied with the different cows from 13 to 26. Sets in which the germ content was more numerous in the back quarters predominated in the case of 9 of the 11 cows. The cases in which the larger germ contents were found in the front quarters were largely confined to the early part of the lactation period or to cases where a front quarter contained large numbers of a particular germ which was practically absent from the corresponding back quarters.

The cause and significance of this unequal distribution of germ life in the front and back quarters of the udder is unknown but the fact is evident from any point of view from which the data has been considered.

Relation of bacterial count to period of lactation.— In searching for the influences which keep the bacteria in the udder within the comparatively narrow bounds which are shown by the preceding data, it will be of interest to observe the relation of the germ content to the period of lactation of the animal. The data were not complete with regard to the period of lactation in a few cases but the results from 1,119 samples are given in Table VII.

Table VII.— Bacteria in Udder at Various Months of Lactation.

	1-5 days.	6-30 days.	2d month.	3d month.	4th month.	5th month.	6th month.	7th month.	8th month.	9th month.	10th month.	11th month.	12th month.
Cows.....	6	13	13	12	8	13	10	13	14	5	9	6	2
Samples.....	48	86	117	113	73	124	74	129	138	80	100	29	8
Total germs...	36,361	21,236	64,214	16,587	3,081	40,853	12,553	13,215	47,571	7,634	4,357	2,598	4,975
Average per cc	757	247	549	147	42	329	170	102	345	95	44	90	622

An examination of the above table shows that, with the exception of the two extremes of the table, there is no evidence of any well marked connection between this germ content and the period of lactation. It will be observed that the samples obtained

during the first five days were separated from those during the remainder of the first month. These first samples were within the colostrum period of flow and a majority of them were obtained at the first milking. The relatively high average of these samples is characteristic of practically all of them and it seems probable that under average conditions the germ content of the colostrum is higher than that of the milk at any later period of lactation.

The high average for the twelfth month, on the other hand, is due to high counts in two of the samples coming from a single cow so that the mathematical average of all of the counts is deceptive. The small numbers found in six of the eight samples during the twelfth month, taken in connection with the results from the two preceding months, suggest that toward the close of the lactation period there is a slight tendency for the germ content of the udder to fall.

It should be observed that these deductions have been obtained by massing together observations without regard to any peculiarities of the individuals from which the samples had been obtained. Deductions from such massed data can well be tested by the results obtained from individual cows. As has been already explained, successive samples were taken over a considerable period from seven cows and a smaller number of samples from 11 others. The results from these individual sets of samples are in almost complete accord with the massed data. The colostrum contains rather high numbers of bacteria, the numbers varying greatly with the different animals. With the secretion of normal milk the numbers fall noticeably and remain reasonably constant during the remainder of the lactation period, usually decreasing slightly toward its close. It should be remembered, however, that even under the most favorable conditions only a single set of three samples was taken once per month and more frequent sampling might disclose minor variations.

It should be further remembered that these statements are intended to apply only to the germ content under normal conditions.

In cases of inflammation of the udder the germ content is frequently, if not always, markedly increased.

Relation of bacterial count to the age of the cow.— The age is unknown in the case of some of the cows whose udder content has been studied, but the results from 1,085 of the samples, arranged according to the ages of the cows, are given in Table VIII.

TABLE VIII.— AVERAGE BACTERIAL CONTENT OF UDDERS OF COWS OF DIFFERENT AGES.

	Two years.	Three years.	Four years.	Five years.	Six years.	Seven years.	Eight years.	Nine years.	Ten years.	Eleven years.
Cows	4	15	25	6	5	10	13	3	2	1
Samples	16	183	365	136	36	116	173	37	19	4
Bacteria per cubic centimeter	99	126	269	233	163	659	750	412	166	2,434

It will be noted that the irregular results shown in years 2, 6, 10 and 11 are associated with a small number of samples from a small number of cows. It is manifest that under such conditions the individual peculiarities of the cows would be most evident. The average of 2,434 for the eleventh year represents four samples from a single cow and cannot be considered as a representative average. This same cow furnished a part of the samples for the preceding year and the average of these samples was 303. The samples from the other cow were even lower so that the average for the tenth year is one of the lowest of the series.

Taken all in all, the above table does not offer satisfactory evidence that the age of the cow has any distinct influence upon the germ content of the udder.

Other factors.— Of the factors influencing the udder content inflammation is one commonly considered in public health work. It is a matter of general knowledge that inflamed udders commonly have high germ contents and such udders have been located through the organisms which are abundant in their secretions. Since inflammation is a pathological condition it has not been intentionally

investigated in connection with this study of the normal udder but its influence may have entered into the results obtained.

In discussing the relation of the germ content to the period of lactation it was observed that during the first few days germs were distinctly more abundant than later and that toward the close of lactation the numbers tended to decrease. It will be remembered that during the first few days the udder is practically always in an inflamed condition. Later, while the normal udder is in full flow, it is daily subjected to complete distention, which is the first stage of inflammation. Finally, when the flow begins to fall off rapidly, the udder is no longer fully distended, and this inflammatory condition is not present. It is noteworthy that the three stages in germ content which were noted (see page 18) are fairly parallel with these three relations to inflammation. In how far this influence of inflammation is responsible for the observed variations in germ content and the method by which its influence is exerted can be best determined after the question of inflammation has been thoroughly studied.

UDDER FLORA.

In studying the flora of any region either of two distinct ideas may be followed; the aim may be to include all of the forms which are ever found in that region or it may be restricted to those which persist in the region for a considerable time and may be considered as fairly permanent residents. In the earlier studies of a flora this latter plan has much to commend it since it is far simpler in execution.

During these qualitative studies samples were obtained under two quite different sets of conditions and for somewhat different purposes.

More than 750 samples were obtained from 15 cows at the station barn where conditions were largely under control and the milkers were accustomed to assisting in the collection of samples. These samples were taken on three successive days from each cow and only those forms were isolated for study which appeared

in two or more samples and in such numbers as to make it evident that they were fairly permanent inhabitants of the udder. There is little reason to doubt but that the forms thus isolated are true members of the udder flora.

Feeling that more extensive observations should be made, 184 samples were obtained on a single occasion from 46 cows in another herd. In this case the samples were drawn into sterile test tubes by either one of us (W) or by Dr. R. S. Breed. Having but a single sample from each quarter of the udder there was no opportunity for observing the persistence of a given form. All plates showing evidence of contamination were rejected and only those types of colonies which were present in considerable numbers were selected for study. While the attempt was made to exclude all contaminating forms there remains the possibility that some of the forms thus selected were not true members of the udder flora.

In any work of this nature the personal element is necessarily large. Success in separating the colonies upon any plate into classes, each of which represents a single group of organisms, requires both experience and judgment. The ability to carry in mind the distinguishing characteristics of a growing list of such classes, so that the colonies present may be correctly assigned to classes which were established months earlier, is especially difficult to acquire. The accuracy of this separation can be tested in a measure by the extended study of representatives of these classes and in this investigation cultures were thus studied in all cases where doubt arose as to the proper classification. However, on account of the large number of colonies which must be classified and the amount of time consumed by the detailed study of selected forms it follows that the classification records must depend largely for their accuracy upon the judgment of the worker.

One of the authors (W) devoted practically his entire attention for approximately one and one-half years to the examination of samples from cows' udders and the classification of the forms found there; and he is to be held personally responsible for the

accuracy of all of the data presented in this portion of the publication.

Method of classification.— The attempt at separating and describing the forms of bacteria which occur in cows' udders meets the difficulty common to an attempt at such a classification in any division of the bacterial flora, the lack of a satisfactory system of classification.

The older system of classification, which was widely applied to this class of bacteria by Conn, Esten and Stocking,[14] proceeded on the assumption that bacteria were especially prone to vary in their physiological activities and accordingly the groups into which they might be arranged must have shadowy and uncertain limits.

The newer system of the Society of American Bacteriologists accepts the division into genera according to the classification of Migula. It proceeds on the assumption that the members of each genus can be reduced to groups, the members of each group agreeing exactly in the possession of or the lack of certain physiological functions. A novel feature in this system is the provision for expressing these functions numerically. By writing these numbers in a definite order the most important physiological activities of the organism in question are expressed tersely and in a form which is most favorable for direct comparison. By arranging many such numerical expressions in order, those with identical physiological functions are grouped together and the numerical expression which characterizes the group becomes their "group number."

While this newer system substitutes scientific accuracy for the hazy generalizations which characterized the older, in its present form it can hardly be considered as fully perfect. Its manifest advantage as a means of tersely recording the observed facts regarding the isolated cultures led to its use in these studies.

The numbers used in recording various important physiological reactions were as follows:

[14] Conn, H. W., Esten, W. M., and Stocking, W. A. A classification of dairy bacteria. Conn. (Storrs) Agr. Exp. Sta. Ann. Rpt. **18** (1906):91–203. 1907.

A Numerical System of Recording the Salient Characters of an Organism.
(Group Number.)

100.	Endospores produced.
200.	Endospores not produced.
10.	Aerobic (Strict).
20.	Facultative anaerobic.
30.	Anaerobic (Strict).
1.	Gelatin liquefied.
2.	Gelatin not liquefied.
0.1	Acid and gas from dextrose.
0.2	Acid without gas from dextrose.
0.3	No acid from dextrose.
0.4	No growth with dextrose.
.01	Acid and gas from lactose.
.02	Acid without gas from lactose.
.03	No acid from lactose.
.04	No growth with lactose.
.001	Acid and gas from saccharose.
.002	Acid without gas from saccharose.
.003	No acid from saccharose.
.004	No growth with saccharose.
.0001	Nitrates reduced with evolution of gas.
.0002	Nitrates not reduced.
.0003	Nitrates reduced without gas formation.
.00001	Fluorescent.
.00002	Violet chromogens.
.00003	Blue "
.00004	Green "
.00005	Yellow "
.00006	Orange "
.00007	Red "
.00008	Brown "
.00009	Pink "
.00000	Non-chromogenic.
.000001	Diastasic action on potato starch, strong.
.000002	Diastasic action on potato starch, feeble.
.000003	Diastasic action on potato starch, absent.
.0000001	Acid and gas from glycerine.
.0000002	Acid without gas from glycerine.
.0000003	No acid from glycerine.
.0000004	No growth with glycerine.

In order to make the results of the present study as intelligible as possible to all workers the probable synonyms in the classification of Conn, Esten and Stocking have been added.

The precautions which were observed in making certain that the forms studied were actually derived from the udder have been explained under the head of technique. In classifying these organisms all determinations were made in triplicate and when for any reason the reaction was in doubt triplicate cultures were again prepared on a later date.

The preparation of the media and the determination of the reactions were in accord with the official methods of the American Public Health Association[15] except that the reduction of nitrate to nitrite was determined by the iodine-starch reaction according to the suggestion of Smith for the reasons given in Technical Bulletin 13.[16]

OBSERVED FORMS.

In accordance with the above systems of classification the observed udder flora may be summarized as follows:

TABLE IX.— LIST OF UDDER BACTERIA.

Cultures Studied.	Culture No.	Society No.	Name.
1	1	Bacterium.....211.2222533	*Bacterium lactis brevis*
1	2	Bacterium.....211.2232523	*Bacterium lactis flocculus*
2	3	Bacterium.....211.2232533	*Bacterium lactis brevis*
2	4	Bacterium.....211.3332513	*Bacterium lactis citronis*
1	5	Bacterium.....212.2223023	*Bacterium lactis non-acidi*
1	6	Bacterium.....212.2232033	*Bacterium lactis non-acidi*
2	7	Bacterium.....212.2233033	*Bacterium lactis non-acidi*
1	8	Bacterium.....212.2233633	*Bacterium lactis aureum II?*
1	9	Bacterium.....212.2322033	*Bacterium lactis non-acidi*
1	10	Bacterium.....212.2332033	*Bacterium lactis non-acidi*
1	11	Bacterium.....212.2332633	*Bacterium lactis aureum II?*
6	12	Bacterium.....212.3332033	*Bacterium lactis Connii?*
2	13	Bacterium.....212.3333033	*Bacterium lactis Connii?*
1	14	Bacterium.....222.2222032	*Bacterium lactis acidi*
3	15	Micrococcus 211.2222533	*Micrococcus lactis varians*
1	16	Micrococcus 211.2222623	*Micrococcus lactis varians A.?*
1	17	Micrococcus 211.2222633	*Micrococcus lactis varians?*
1	18	Micrococcus 211.2222922	*Micrococcus lactis varians*
3	19	Micrococcus 211.2223032	*Micrococcus lactis albidus*
8	20	Micrococcus 211.2223033	*Micrococcus lactis albidus*
4	21	Micrococcus 211.2223532	*Micrococcus lactis varians?*
2	22	Micrococcus 211.2223533	*Micrococcus lactis varians?*
1	23	Micrococcus 211.2223623	*Micrococcus lactis rugosus*
6	24	Micrococcus 211.2223633	*Micrococcus lactis varians?*
2	25	Micrococcus 211.2223733	*Micrococcus lactis rubidus?*
1	26	Micrococcus 211.2223923	*Micrococcus lactis rugosus*
3	27	Micrococcus 211.2223933	*Micrococcus lactis rugosus?*
1	28	Micrococcus 211.2232033	*Micrococcus lactis albidus*
9	29	Micrococcus 211.2233033	*Micrococcus lactis albidus*
1	30	Micrococcus 211.2233533	*Micrococcus lactis varians?*
1	31	Micrococcus 211.2323032	*Micrococcus lactis albidus*
4	32	Micrococcus 211.2323033	*Micrococcus lactis albidus?*

[15] Amer. Public Health Association. Report of the committee on standard methods for the bacterial analysis of milk. *Amer. Jour. Pub. Hyg.* **20**:315-345. 1910.

[16] Harding, H. A. The constancy of certain physiological characters in the classification of bacteria. N. Y. (State) Agr. Exp. Sta. Technical Bul. 13. 1910. Also in Ann. Rpt. of same Station **29** (1910):55–93. 1911.

TABLE IX (*concluded*).

Cultures Studied.	Culture No.	Society No.	Name.
1	33	Micrococcus 211.2323532	*Micrococcus lactis varians?*
1	34	Micrococcus 211.2323533	*Micrococcus lactis varians?*
1	35	Micrococcus 211.2323932	*Micrococcus lactis rugosus?*
1	36	Micrococcus 211.2332523	*Micrococcus lactis varians?*
1	37	Micrococcus 211.2333033	*Micrococcus lactis albidus?*
1	38	Micrococcus 211.3233033	*Micrococcus lactis albus?*
1	39	Micrococcus 212.2222023	*Micrococcus lactis gigas?*
2	40	Micrococcus 212.2222033	*Micrococcus lactis acidi*
1	41	Micrococcus 212.2222532	*Micrococcus lactis aureus*
1	42	Micrococcus 212.2222533	*Micrococcus lactis aureus?*
1	43	Micrococcus 212.2223032	*Micrococcus lactis acidi*
1	44	Micrococcus 212.2223532	*Micrococcus lactis aureus*
5	45	Micrococcus 212.2223033	*Micrococcus lactis acidi*
1	46	Micrococcus 212.2223523	*Micrococcus lactis aureus*
1	47	Micrococcus 212.2223533	*Micrococcus lactis aureus?*
1	48	Micrococcus 212.2232033	*Micrococcus lactis acidi?*
1	49	Micrococcus 212.2232533	*Micrococcus lactis aureus?*
1	50	Micrococcus 212.2323033	*Micrococcus lactis acidi?*
1	51	Micrococcus 212.2333533	*Micrococcus lactis aureus?*
5	52	Micrococcus 212.3332033	*Galactococcus versicolor*
4	53	Micrococcus 212.3333033	*Galactococcus versicolor*
3	54	Micrococcus 212.3333533	*Micrococcus lactis citreus*
1	55	Micrococcus 212.3333633	*Micrococcus lactis flavus?*
1	56	Micrococcus 221.2222032	*Micrococcus lactis albidus?*
1	57	Micrococcus 221.2222622	*Micrococcus lactis varians?*
1	58	Micrococcus 221.2222632	*Micrococcus lactis varians*
1	59	Micrococcus 221.2223023	*Micrococcus lactis albidus*
4	60	Micrococcus 221.2223033	*Micrococcus lactis albidus*
1	61	Micrococcus 221.2223513	*Micrococcus lactis varians?*
3	62	Micrococcus 221.2223532	*Micrococcus lactis varians?*
1	63	Micrococcus 221.2223533	*Micrococcus lactis varians?*
1	64	Micrococcus 221.2223632	*Micrococcus lactis varians?*
1	65	Micrococcus 221.2233633	*Micrococcus lactis rugosus?*
2	66	Micrococcus 222.2222034	*Streptococcus lacticus?*
2	67	Micrococcus 222.2223533	*Micrococcus lactis aureus*
1	68	Streptococcus...211.2223033	*Micrococcus lactis albidus?*
1	69	Streptococcus...222.2223033	*Streptococcus lacticus?*
1	70	Yeast.....212.2332013	
1	71	Yeast.....212.2332033	

The finding of only 71 distinct groups of organisms in a study of over 900 samples of milk from the udder suggests that there is a fairly small and distinct flora of this region which recurs in the various samples.

The showing made by the older system of classification is even more striking since only about one-fourth as many species are represented. This decrease in number of species, however, is more apparent than real since barely 10 per ct. of the cultures were typical members of the groups described by Conn, Esten and

Stocking. Nearly one-half of them differed so materially from the types described by the above authors that they were listed under their nearest allies with the addition of an interrogation point for the double purpose of indicating their general relationship and of avoiding the coinage of any more names. The remaining 40 per ct. come more or less clearly under the shadowy outlines of the types of Conn and his colleagues.

Under the heading "cultures studied" is given the number of cultures which were studied in detail and whose group number was fully determined. A larger number of cultures were given a preliminary study, sufficient to convince the worker that the culture finally studied in detail was a true type of the class of colonies which it was taken to represent. The extended study of a considerable number of cultures of the same group indicates not so much the frequency with which the cultures were encountered as the variation in colony appearance which rendered the identity doubtful in the mind of the observer.

The present group number is not perfect and in some cases two or more really distinct strains are undoubtedly included in a single group. The probability of such inclusion is greater in the groups having dissimilar colonies as noted above. It should be noted that whatever criticism is directed against the Society classification system because it does not separate all distinct strains bears even harder on the older classification since none of these collections of cultures which were grouped together by the Society system were separated by the older grouping.

The udder organisms undoubtedly enter through the teat opening and advance into the finer subdivisions of the udder at times against the force of gravity and the flow of milk. Under such circumstances it would seem that swimming organs would be a contributing factor in their struggle for existence. Since the genus bacillus — motile rods — is absent from the above list of udder organisms it is probable that flagella are correlated with something else which is distinctly unsuited to udder conditions.

The genus Streptococcus is also conspicuous because only two

representatives were found. The absence of this genus was noted early in the qualitative work and attention given to the detection of streptococci. Neither of the two forms here listed are typical members of the genus, their tendency to form chains being rather weak. It will also be noted that two strains of *Streptococcus lacticus* are among the named species with an interrogation point. These strains were not streptococci but agreed fairly closely with the description of that species in other respects. Many of the streptococci, which have been isolated from other sources, grow rather poorly upon the standard agar used in this study and it is possible that additional forms would have been found here had special media[17] been employed in the search.

The two non-spore-bearing yeasts were also unexpected forms but were so constantly present in large numbers in at least one udder as to be undoubted members of the udder flora. No spores were observed in these yeasts even when grown on gypsum blocks.

The greater number — 75 per ct.— of these forms were included in the genus Micrococcus. This is quite in accord with the experience of other students who have found udder cocci especially abundant.

There is an entire absence of spore formation among the organisms here described as representatives of the udder flora. This is not so unusual as would at first appear since 77 per ct. of these forms are cocci, among which spore formation is unknown.

Strict anaerobes are lacking in the above list since the technique employed would not have shown those which may have been present. Facultative anaerobes make up 20 per ct. of the list and the remaining 80 per ct. are classed as strict aerobes. This classification of the oxygen relation of the forms was made on the basis of their growth in the closed arm of the fermentation tube. Either this basis of classification is not accurate or the aeration within the udder is vastly different from what one would expect from the anatomy of the organ. If free oxygen is absent

[17] North, C. E. An agar gelatin medium. *Jour. of Med. Research* 20:359–363. 1909.

from the interior of the udder, as is commonly supposed, it is difficult to understand why the major portion of the udder flora consists of forms which cannot thrive under anaerobic conditions in laboratory cultures.

Gelatin is liquefied by 55 per ct. of the above list of germs, although the action is rather slow in most cases. It is ordinarily assumed that the ability to attack gelatin carries with it the ability to digest casein. This latter ability is noted from the appearance of tubes of inoculated milk. This method is crude and unsatisfactory in cases where the action is slow. In the present instance the digestion of casein was thus noted in only one-half of the groups where gelatin was liquefied. There is a strong probability that if sufficiently delicate tests of the action on casein had been applied the numbers digesting casein and gelatin would have been practically equal. The standard technique is much in need of improvement at this point. The most helpful suggestion for improving the technique is that of Hastings.[18]

With a large part of the udder flora provided with enzymes capable of digesting casein the milk within the udder should be attacked to a noticeable extent. In colostrum the changed condition of the milk is quite noticeable but so far as known this change has not been shown to be due to bacterial action. In the milk as normally drawn there is a measurable amount of water-soluble nitrogen and the extent to which these water-soluble nitrogenous compounds are derived from normal milk by bacterial action within the udder remains to be studied.

A considerable number of enzymes have been found in cows' milk in recent years. It remains to be shown to what extent these enzymes are secreted by the cow and how far they are elaborated by the bacteria which chance to be dwelling within the particular udder. In Bulletin 203[19] attention was called to an experimental

[18] Hastings, E. G. The action of various classes of bacteria on casein as shown by milk agar plates. *Cent. f. Bak.* II Abt. **12**:590–592. 1904.

[19] Van Slyke, L. L., Harding, H. A., and Hart, E. B. A study of enzymes in cheese. N. Y. (State) Agr. Exp. Sta. Bul. 203. 1901. Also in Ann. Rpt. of same Station **20** (1901):165–193. 1902.

demonstration of the correlation between the germ content of quarters of an udder and the rate at which milk from these quarters underwent auto-digestion in the presence of chloroform. These results were not intended as a demonstration that all of the enzymes in milk were derived from bacteria but rather as a suggestion that bacterial enzymes within the udder are a factor which must be considered in studying true milk enzymes.

The fermentative activity of the udder flora is especially interesting. No visible gas is formed from dextrose, lactose, saccharose or glycerine when tested in the fermentation tube. This is not due to inability to attack these substances since acid was formed from dextrose by 89 per ct., from lactose by 70 per ct., from saccharose by 63 per ct. and from glycerin by 21 per ct. of the forms studied.

The technique employed in determining the reduction of nitrates has been already described. Nitrates were reduced by 59 per ct. of the forms but in no case was gas detected in the fermentation tube as a result of the reduction.

The records of color are particularly unsatisfactory in connection with these forms. While the difficulty is partly due to the tardy formation of slight amounts of coloring matter it is more largely due to the fact that the color is frequently salmon pink. Under varying conditions of age, light, moisture and substratum there is a tendency to classify cultures of the same organism under yellow, orange, red, brown and pink. The standard method of classification needs improvement at this point so that direct comparison can be made with standard test objects, using cultures of definite age and composition. The color given in the above table is that observed on the agar slope at the end of two weeks.

The action of the organism upon starch was judged by crushing fourteen-day potato cultures in a mortar and testing for starch with a dilute iodine solution. Where the action upon the starch was slight the fact was often best determined by adding the iodine solution to the fluid from the potato culture. The presence of the first products of starch digestion was shown by a wine color.

The lack of even the wine color was taken as evidence that the starch had not been attacked. This was the case with 80 per ct. of the cultures. Of the remainder the starch was practically all destroyed in 4 per ct. and 16 per ct. were classed as feeble. The separation of the cases where starch is attacked into two classes is unsatisfactory, since there was no available turning point between those where the action was barely perceptible and where it was practically complete.

Among the observed reactions which were not enumerated in Table I, the results with Gram stain are the most striking. With the exception of a single culture, No. 28, all of the isolated cultures were tested in this way and all but six gave positive reactions. The reaction of the single strain under No. 30 was variable and that of the single strains representing No. 1 and No. 2 was negative. One of the two strains under No. 7, one of the six strains under No. 12 and one of the three strains under No. 54 were also negative.

This proportion of Gram positive strains, 96 per ct., is surprisingly large and may be taken as one of the general characteristics of the udder flora.

Relative frequency of occurrence.— Some forms were observed in only a series of samples from a single cow while others were common in samples from various sources. In some cases the bacteria in a given quarter were few and the flora mixed while in others they were abundant and often consisted of practically a single form. These conditions can perhaps be best illustrated by tabulating the forms recognized in the case of the fifteen cows from the Station herd. These data are given in Table X.

TABLE X.

Date.	Cow.	R. F.	L. F.	R. B.	L. B.
2- 7-11	Anna G.	29*			
2- 8-11		29			
2- 9-11		29			
2- 7-11	Carey D. H.....		18, 15	61, 36	
2- 8-11		15, 26	18, 15	15	15, 39
2- 9-11		15	18, 15	16, 49, 71	39, 23

* The numbers given in this and the following tables are the culture numbers given in Table IX.

TABLE X (*continued*).

Date.	Cow.	R. F.	L. F.	R. B.	L. B.
2- 8-11	Carey F.		15	15	
2- 9-11				15	15, 63
5- 8-11				21	
5- 9-11				21	
5-10-11				21	21
5-11-11				21	
5-12-11			21	21	21
5-13-11				21	21
5- 8-11	Carey S.	49	49	12	12
5- 9-11		49, 3		12	
3- 8-10	Gertie F. No. 2.....	29			48, 17
3- 9-10				29	9, 29, 17
3-10-10			2, 29		29, 17
4-12-10		30			
4-13-10		30	24		
4-14-10			24, 30		30
5-11-10		29			
11- 9-10			24	**30**	**24**
11-10-10			**24**	24	**24**
11-11-10			**24**	**17**	**24**
12- 7-10			24		24
12- 8-10			24		24
12- 9-10			24		2
1-11-11				24	24
1-12-11				2	24
1-16-11				2	24
2-14-11				29, 25	27
2-15-11			25	29, 25	24
2-16-11		25	25	25	24
2- 7-11	Gertie F. No. 3.....	46	24		
2- 8-11			70 or 71		24, 70 or 71
2- 9-11		25, 70 or 71			
5- 8-11		**33**			
5- 9-11		**33**			
5-10-11		**33**			
5-11-11		33			
5-12-11		**33**			
5-13-11		**33**	33		
3- 2-11	Hammond	**53**	**19**	**53**	**53**
5- 9-11		**53**	**19**	**53**	**53**
5-10-11		**53**	**19**	**53**	**53**
5-11-11		**53**	**19, 53**	53	**53**
5-12-11		**53**, 56	**19**	**53**	**53**
5-13-11		**53**		**53**	**53**
2- 8-11	Hammond F. No. 2....	3			
5- 8-11					60
5- 9-11					60
5-10-11		21			60
5-11-11		60, 33		60	60
5-12-11					60
5-13-11					60
3- 8-10	Mabel S. F.....	**29**	29, 17	24, 17, 29	**29**
3- 9-10		**29**, 24	**29**, 17, 24		
3-10-10			17, 24		29
4-12-10		**29**, 24	29, 24	**24**	

TABLE X (*continued*).

Date.	Cow.	R. F.	L. F.	R. B.	L. B.
4-13-10	Mabel S. F.....	29	24	29	29
4-14-10		**29**	**24**	**29**	
5-10-10			24	**29**	29
5-11-10			24, 30	**29**	
5-12-10		**29**	24		
7-20-10		24, 29	29		29
7-21-10		24, 29	29	29	
7-22-10		29	29	24	
3- 8-10	Millie D.	**29**	6	**29**	65
3- 9-10					65, 6
3-10-10				24	**29**
5-31-10		6, 54	6, 52	6, 54	6
6- 1-10		6	6	6	6
6- 3-10				6	
6-20-10		52, 54	13	6	6
6-21-10		13	13	6, 59	**6**
6-22-10		52, 6, 24	52, 59	6	6, 24
11- 9-10		52, 17		6	**6**, 17
11-10-10		**52**, 17	52, 17	6	52
11-11-10		**6**	**6, 2**	**6**	2
12- 7-10		**29**, 54	**29**	**29**	**6, 29**
12- 8-10		**54**	**54**	**29**	**54**
12- 9-10		**54, 29**	**54**	**29**	**54**
1-11-11		54			54, 29
1-12-11		2, 29		**29**	54
1-16-11		**58**		6	**2**, 1
2-14-11		**29, 27**	**29, 27**	**29, 27**, 8	**29**
2-15-11		**29, 27**	**29, 27**	**29, 27**	**29**
2-16-11		**29, 27**	**29, 27**	**29**	**29, 27**
3- 8-10	Millie F.	24, 9	9		7, 29
3- 9-10		17, 24, 7			6, 30, 17, 24
3-10-10		7, 24	24	9	65
4-12-10		24, 53		6	17, 7
4-13-10		30, **52**	**52**	**7**	30
4-14-10		24, 9	**52**	9, 24	**65**
5-10-10		7, **52**	**52**	30, **29**	65, 6
5-11-10		**52**	**52**	**29**, 30, 24	65
5-12-10		**52**	**52**, 65, 24	**29**	65, 24
7-20-10		53, 30		38	7
7-21-10				38	7
7-22-10		53	53	38	7
3- 8-10	Millie F. B. B.....	24		29, 6	29, 24
3- 9-10			30	17	
3-10-10		9, 24		9, 30	65
4-12-10				9	29
4-13-10		24			
4-14-10		24	24	24, 30	65
2- 7-11	Millie G.	**37**	71		
2- 8-11		**37**			71
2- 9-11		**37**	20, 63		71
2-16-11		**37**			71
5- 8-11		**19**	19	19	71
5- 9-11		19		19	71
5-10-11		19		19	71
5-11-11		19		19	**71**

TABLE X (*concluded*).

Date.	Cow.	R. F.	L. F.	R. B.	L. B.
5-12-11	Millie G.	19		19	71
5-13-11		**19**		19	**71**
3- 8-10	Ruth F.	6			24
3- 9-10					24
3-10-10			17, 66	9, 66	24, 9
5-26-10			66		24
6-20-10					24
7-21-10		9	20	17, 66	24
7-22-10		6	66	6	24
11- 9-10		6	6	**6**, 17	24
11-10-10		6, 24	6	**6**	24
11-11-10		**6**	24	**6**	24
12- 7-10				**6**	24
12- 8-10				**6**, 17	24
12- 9-10				**6**, 17	24
1-11-11			2	6, 24	24
1-12-11				24	24
1-16-11				6, 29	24
2-14-11				**29**, 27	24, 27
2-15-11				**29**, 27	24, 27
2-16-11			28	**29**, 27	24, 27
4-12-10	Ruth S.	30		52, 30	
4-13-10		30		52, 30	
4-14-10		30, 29		52, 13	13
5-10-10		**52**		**52**, 30	13, 9
5-11-10		9		13	13, 7, 9
5-12-10		**7, 9**		**13, 52**	13, 7, 9
7-20-10		66		66	66
7-21-10		66, 30		66	66
6-22-10		66		66	66

The above table shows that 45 groups of organisms were recognized in 750 samples representing 15 cows. The frequent failure to classify the organisms, particularly in the front quarters, was largely due to the small numbers present and the consequent uncertainty as to whether the colonies on the plates represented real members of the udder flora. Occasionally the attempt at classification was omitted because of the mixed character of the growth and suspicion that the sample may have suffered from some unknown contamination. A few of the vacancies are due to the loss of type cultures before their study had been completed. In a fair proportion of cases miscellaneous colonies appeared upon the plates. They were not included in the classification because, as already explained, every effort was directed toward restricting the classified forms to members of the true udder flora.

Ruth S. gave no milk from the left front quarter of her udder.

It will be noted that in one or more quarters of many of the cows a single organism persisted for long periods and during this time it was the predominating organism in most cases. These observations offer the strongest evidence for the inference that such forms have so adapted themselves to the conditions found in the interior of the udder as to be considered fairly permanent inhabitants. Such being the case it is hard to understand why they do not attain much larger numbers than have been observed during these studies.

It will also be observed that in those cases where a given form is abundant in one quarter it is at least occasionally met in the milk from the other quarters. This may be explained on the basis that it was really present all of the time but was missed in the cultures. While this may have been the case in some instances, the use of one cubic centimeter sample of milk at each examination should have reduced this difficulty very markedly since an organism which is present in a proportion of less than one organism to a cubic centimeter of milk is surely not very abundant. On the other hand, the spreading of the germs from one quarter to another may have been connected with the ordinary milking process. While it is the aim of most milkers to avoid wetting the hand, they are frequently moistened, especially in the act of stripping. With hands thus moistened the infection of the exterior of one teat with the germs from the other quarter is relatively easy.

The yeast culture was so abundant and so constant in the left back quarter of Millie G. as to put at rest any doubt as to its being a member of the udder flora.

While the persistence of a given group in a single udder, as well as its distribution among the various quarters of an udder, is well shown in Table X, the distribution of the various groups among these fifteen cows is better shown in Table XI.

TABLE XI.— DISTRIBUTION OF OBSERVED BACTERIA AMONG THE UDDERS OF DIFFERENT COWS.

Anna G.	Carey D. H.	Carey F.	Carey S.	Gertie F. No. 2.	Gertie F. No. 3.	Hammond No. 2.	Hammond F. No. 2.	Millie D.	Millie F.	Millie F. B. B.	Millie G.	Mabel S. F.	Ruth F.	Ruth S.
				2				1						
			3				3	2					2	
								6	6	6			6	
									7					7
								8						
				9					9	9			9	9
			12											
								13						13
	15	15												
	16													
				17				17	17	17		17	17	
	18													
						19					19			
							21				20		20	
		21												
	23													
				24	24			24	24	24		24	24	
				25	25									
	26													
				27				27					27	
													28	
29				29				29	29	29		29	29	29
				30					30	30		30		30
					33		33							
	36													
											37			
									38					
	39													
					46									
				48										
	49		49											
								52	52					52
						53			53					
								54						
						56								
								58						
								59						
							60							
	61													
		63									63			
								65	65	65				
					70								66	66
	71				71						71			

The numbers given in the above table correspond to the culture numbers used in the list of udder bacteria in Table IX and it is

believed that these 45 groups can be safely considered as true members of the udder flora.

It is seen that Nos. 6, 9, 17, 24, 29 and 30 were obtained from the udders of four or more of the 15 cows. Expressed in the older nomenclature the first two of these are *Bacterium lactis non-acidi,* the next two are variant strains associated with *Bacterium lactis varians,* No. 29 is *Bacterium lactis albidus* and the last is also *Bacterium lactis varians.* Of these cultures Nos. 29, 24 and 17 were the most common, having been found in 8, 7 and 6 udders, respectively.

The cultures isolated from the samples obtained on a single occasion from another herd cannot all be assigned to the udder flora with the same assurance as those given above, but the certainty of their having been derived from this source is increased in proportion to the number of udder samples in which they were observed.

The culture numbers noted in the 184 samples from the herd of Mr. A. G. Lewis and the number of samples in which each was found are given in Table XII.

Table XII.—Frequency of Isolation of Udder Forms from 184 Samples.

Culture No.	Times found.	Culture No.	Times found.	Culture No.	Times found.
4	2	32	16	**51**	**1**
5	1	34	2	**52**	**1**
10	2	35	3	**53**	**5**
11	1	40	5	**54**	**2**
12	**6**	41	6	55	2
13	**1**	42	5	57	1
14	1	43	1	62	**7**
20	16	44	1	64	4
22	6	45	6	**67**	**3**
24	5	47	3	69	**1**
29	20	**49**	7	**70**	**1**
31	4	50	1		

The culture numbers given in bold face in the above table are those previously noted in Table XI. Finding them in the udders in this separate herd, many members of which had been recently imported from the Island of Guernsey throws some light upon their international distribution. Of the forms not previously encountered a number of them occurred in so many samples there can be no doubt of their being true members of the udder flora. The most doubtful forms are those found in but one or two samples. They would have been excluded from the list had any suspicious circumstance been observed in connection with their isola-

tion. As matters stand they cannot be accepted as members of the udder flora with the same feeling of certainty which accompanies the other forms.

Temperature relations.— By referring to Table X it will be seen that certain numbers in the table are printed in bold face type, which signifies that these forms did not appear on the plates kept five days at room temperature but did appear after these plates had been held two additional days at 37° C. This would indicate that these particular forms had become so habituated to the blood heat of the cow as to grow rather poorly at lower temperatures.

The distribution of this class of organisms is also interesting. They are lacking in Carey F., Hammond F. No. 2 and Millie F. B. B., make up the entire flora in Hammond No. 2 and are present in varying proportions in the other udders.

A partial explanation of this temperature relation is found by studying the udders in which there is a mixed result, as with Millie D. Here the examinations extended over a year and it will be observed that the additional growth at 37° C. was mainly during the colder portions of the year. While the temperature of the room in which the plates were held for five days was maintained at approximately 70° F. during the day the temperature fell sharply at night with the result that the growth of these udder germs was markedly checked. These irregular results due to incubation at room temperature emphasize the weakness of this procedure and the need of a low temperature incubator such as has been constructed and will be described in a forthcoming bulletin. (Technical Bulletin No. 29.)

It is clear, however, that the above is not an entire explanation of this temperature relation since the tests with Carey F., where the high-temperature-loving forms were absent and with Hammond No. 2, where they made up the entire flora, were made simultaneously.

RELATION OF UDDER FLORA TO MILK PRODUCTS.

Practically the only extended study of milk products which is available for direct comparison is that of the flora of cheddar cheese as given in Technical Bulletin 8.[20] At that time the Society

[20] Harding, H. A., and Prucha, M. J. The bacterial flora of cheddar cheese. N. Y. (State) Agr. Exp. Sta. Technical Bulletin 8. 1908. Also in Ann. Rpt. of same Station **27** (1908): 48–120. 1909.

group number contained only eight places instead of ten as at present.

Making due allowance for this difference in the basis of classification 15 of the 71 groups from the udder are probably comparable to those found in cheddar cheese. It is noteworthy that none of these 15 groups are among those most abundant in the udder and only six of them, Nos. 52, 53, 54, 59, 60 and 63 were among the groups given in Table XI.

Perhaps the best known of these 15 groups are Nos. 40 and 69 which are typical organisms of sour milk. It should be stated that neither of these organisms was found in any of the more than 700 samples from the Station cows. On the other hand No. 40 was found in five and No. 69 in one of 184 samples taken from another herd. The fact that this large number of samples was taken in a strange barn at a single milking suggests the possibility of accidental contamination. Nothing was observed in connection with the sampling to suggest contamination nor was it evident from the colony growths aside from the unusual physiological properties of the organisms present. The finding of such acid-producing organisms in the udder is not new, similar observations having been made by von Freudenreich,[21] Gorini,[22] and Harrison.[23]

It should be noted in this connection that it was a common practice, in the herd in which these acid organisms were recovered from the udders, to permit the cows to be suckled for varying lengths of time by calves. Esten[24] has pointed out that the mouth of the cow is a habitat of acid organisms and it is possible that the mouth of the calf was the source of these acid organisms recovered in the udder samples.

A more detailed study of the later history of milk and its products must be made before the influence exerted by the udder flora

[21] v. Freudenreich, Ed. Ueber die Bakterien in Kuheuter und ihre Verteilung in den verschiedenen Partien des Melkens. *Landw. Jahrb. Schweiz.* **18**:401–427. 1904. Also given in Sur les bactéries de la mamelle de la vache et sur la manière dont elles sont réparties dans les différentes portions de la traite. *Rev. gen. Lait.* **3**:416–425; 440–448; 462–473. 1904.

[22] Gorini, C. Sui Bacteri dei dotti galattofori delle vacche. *Atti della R. Accademia dei Lincei*, Series 5ª Rendiconti, **11**: (2nd Semestre): 159–165. 1902.

[23] Harrison, F. C., and Savage, A. The bacterial content of the normal udder. *Rev. gen. Lait* **9**:121–131. 1912.

[24] Esten, W. M. Bacterium lactis acidi and its sources. Conn. (Storrs) Sta. Bul. 59. 1909.

upon the later germ life in the milk can be definitely stated. So far as the data are now available the germ life in the udder does not maintain itself very successfully in the competition which it meets after being drawn. This is to be expected on account of the sudden change in environment to which it is subjected. The oxygen within the udder must be scanty if not absent and the temperature is above 37° C. After being drawn the oxygen supply is markedly increased while the temperature is usually rapidly decreased.

While the udder flora as a whole probably does not persist in the milk after it is drawn, some portions of it may do so. The observations on cheddar cheese as quoted above make it probable that a number of forms actually do persist for a considerable period. Gorini[25] has ascribed to the acid-forming liquefiers of the type of *M. lactis varians* a leading role in the ripening of hard cheese. If this supposition is correct, it is evident that the udder flora exerts a marked influence upon this important industry.

It should not be assumed that with the disappearance of the udder forms their influence upon the milk is entirely dissipated. The work of Marshall and his students[26] has shown that even after the total destruction of a given form it may leave such an impress upon the milk as to affect markedly the growth of the organisms which succeed it.

It should also be remembered that the present study has not taken account of the anaerobic flora of the udder, if such exists. The natural lack of aeration in the udder is so great that the conditions there would seem ideal for the development of a distinctive anaerobic flora.

It is much to be regretted that this study has not been more complete along a number of lines but the removal of each of the authors to other fields of activity has at least temporarily halted the investigation.

CONCLUSIONS.

Many of the forms found in the udder are so accustomed to relatively high temperatures that they do not develop satisfactorily at ordinary room temperatures. This fact should be con-

[25] Gorini, C. Ueber die saure-labbildenden Bakterien der Milch. *Cent. Bak.* II Abt. 8:137–140. 1902.

[26] Marshall, C. E. Additional work upon the associative action of bacteria in the souring of milk. *Cent. Bak.* II Abt. 12:593–597. 1904.

sidered in connection with any study of the milk flora but its importance is probably greatest in samples of freshly drawn milk.

Bacteria are most abundant in the first few streams or foremilk, are distinctly less abundant during the main portion of the milking and again become more abundant in the strippings. As a result of this relationship a fairly close approximation of the germ content of the entire flow of milk can be obtained by examining the strippings. Samples taken at this stage of the milking are especially satisfactory as a basis for study of the udder flora.

The germ content was found to be unequally distributed in the udder, the back quarters having about three times as many germs per cubic centimeter as the front quarters. The average of 1,230 samples was 428 per cc., but as the back quarters furnish more milk than the front the average germ content in milk which can be ascribed to the udder content will be approximately 500 per cc. Only 8 per ct. of the samples had a germ content of over 1,000 per cc.

The connection between the period of lactation and the germ content was not very marked. The colostrum showed a slightly higher and the milk of the twelfth month a slightly lower content than the intervening periods.

The age of the cow likewise was not found to exert any appreciable effect upon the germ content of the udder.

The kinds of organisms present in over 900 samples of milk were studied and 71 groups described as members of the udder flora. No organisms producing spores and no motile forms were found. Seventy-five per ct. of the forms were micrococci but only two streptococci were isolated. The need of free oxygen was so great that 80 per ct. of the forms were not able to produce turbidity in the closed arm of the fermentation tube. Gelatin was liquefied by 55 per ct. of the forms and digestion of milk was evident in cultures of about one-half of these forms, the remainder probably liquefying too slowly to be determined by this comparatively crude method. No gas was formed in fermentation tubes in the presence of dextrose, lactose, saccharose or glycerin but acid was formed in percentages varying from 89 to 21 per ct. of the forms with the different sugars. Nitrates were reduced by 59 per ct. of the forms and starch was attacked by 20 per ct. The Gram stain was positive with 96 per ct.

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GENEVA, N. Y.

ZINC ARSENITE AS AN INSECTICIDE

W. J. SCHOENE.

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Address all correspondence, not to individual members of the staff, but to the NEW YORK AGRICULTURAL EXPERIMENT STATION, GENEVA, N. Y.

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*Riverhead, N. Y. †Absent on leave. ‡Connected with the Chautauqua Grape Work.

TECHNICAL BULLETIN No. 28.

ZINC ARSENITE AS AN INSECTICIDE.

W. J. SCHOENE.

SUMMARY.

This is a report of a series of experiments with zinc arsenite and lead arsenate to determine their relative toxicity to insects and the safeness of zinc arsenite for use on foliage.

One pound of zinc arsenite proved equal in effectiveness to three pounds of lead arsenate.

Zinc arsenite when added to calcium hydrate or bordeaux mixture caused no injury to apple foliage; but more or less spotting of apple leaves occurred when the poison was used singly or in combination with lime-sulphur or glucose. Zinc arsenite alone or with glucose caused severe burning of grape foliage. Laboratory tests suggest that the injury to foliage by zinc arsenite may be due in part to the solubility of the poison in carbonic acid.

The contradictory results from the use of zinc arsenite on foliage suggest that the poison as manufactured is not a stable or uniform product.

Zinc arsenite or lead arsenate with bordeaux, soap or glue continued effective for twenty-five days. Either of the poisons alone or with glucose gradually lost its poisonous properties on exposure to weather and by the end of this period had ceased to protect the foliage.

Incidental to the main problem it appears in these tests that the lime-sulphur solution does not resist wet weather as well as bordeaux mixture.

INTRODUCTION.

Zinc arsenite has recently been introduced as an insecticide and has been advertised for sale in this State. The chief advantages claimed for the poison are that it has a high arsenical content and is in a form readily available to insects. On the basis of the prevailing market prices it is, besides, more economical than arsenate of lead, which at the present is largely used in spraying operations against leaf-eating and fruit-eating insects. The experiments herein reported were planned to determine by field and laboratory tests its rate of action as compared with arsenate of lead, and its safeness

for use on foliage; and to ascertain its relative adhesive qualities under ordinary weather conditions.

PREVIOUS REPORTS ON ZINC ARSENITE.

Luther[1] states that in the Pajaro Valley, California, zinc arsenite can be used with safety on the foliage of apple, potato and bean, but not on peach. Woodworth[2] in speaking of spraying operations in the same region says that "this [zinc arsenite] has proven to be the safest of the arsenicals that can be procured in the form of a dry powder" and that "there is no doubt that the zinc arsenite stands foremost at the present time among the available arsenicals with high arsenic content." Swingle and Morris[3] in a study of bark injury to fruit trees caused by arsenicals state that "zinc arsenite (ortho) gave practically no injury under the most severe conditions of the tests . . . ". Cooley[4] reports its successful use against the potato beetle (*Leptinotarsa decemlineata* Say), the larva of the European cabbage butterfly (*Pieris rapæ* L.) and the diamond-back moth (*Plutella maculipennis* Curt.), without injury to the foliage of potato or cabbage. Johnston[5] reports favorable results in a potato-spraying experiment carried on in cooperation with the Virginia Truck Experiment Station. In marked contrast to the above accounts is the report of Britton[6] to the effect that two sprayings at an interval of seven days using three-fourths of a pound of ortho zinc arsenite to fifty gallons of water were followed by severe injury to apple, which caused about one-half of the leaves to drop. Volck[7] recommends the substitution of zinc arsenite for arsenate of lead in the first two sprayings for the codling moth and he further states that "zinc arsenite . . . may cause foliage injury if not properly handled. When mixed with iron sulphide the foliage-injuring properties are largely restrained, and several applications may be made [to apple]. When no iron sulphide is used only the first (full bloom) spraying should be applied with this arsenical." Gillette[8] says that during 1911 zinc arsenite was used by a number of fruit growers on the western slope and no damage resulted except from a single lot of the poison. He suggests that the zinc arsenite manufactured is perhaps not of uniform quality. Melander[9] says that in his tests zinc arsenite has not caused injury to the foliage of apple.

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[1] *Better Fruit*, **5**, No. 8: 65–66. 1911.
[2] *Better Fruit*, **5**, No. 8: 70. 1911.
[3] *Phytopathology*, **1**: 93. 1911.
[4] *Jour. Econ. Ent.*, **5**: 142–6. 1912.
[5] U. S. Dept. Agr. Ent. Bul. 109, pp. 53–65. 1912.
[6] Conn. State Exp. Sta. Ann. Rept. 1911, p. 358.
[7] *Better Fruit*, **6**, No. 11: 46–47. 1912.
[8] Letter of June 19, 1912.
[9] *Better Fruit*, **6**, No. 12: 13. 1912.

STATION EXPERIMENTS WITH ZINC ARSENITE.

CONDITIONS OF THE EXPERIMENTS.

Tests to determine the effects of weather on zinc arsenite and its safeness for use on foliage were conducted in the field while most of the feeding tests were performed in the laboratory under conditions that made it possible to observe closely the behavior of the poison upon the insects. The feeding tests were made by supplying sprayed foliage to a definite number of insects enclosed in cages. The leaves and twigs used in most of these experiments were sprayed in the laboratory with an aspirator. To determine the lasting properties of the mixtures when exposed to weather, trees were sprayed in the field from which at intervals foliage was removed and fed to insects. If there was only a small consumption of foliage the amount of feeding of the insects was estimated in square centimeters, but if the amount consumed was large it was approximated and designated by such terms as moderate, extensive, etc.

In addition to these laboratory studies, field tests were made in the Station nursery and orchard by spraying trees upon which the insects were already established, to compare the action of the poisons on host and parasite under out-of-doors conditions. To each fifty gallons of the diluted spray mixture one of the poisons was added as follows: zinc arsenite one pound, lead arsenate (paste) three pounds, or lead arsenate (dry) one and one-half pounds. The zinc arsenite contained 40.28 per ct. arsenious oxide, and the arsenate of lead paste 17.75 per ct. arsenic oxide.[1]

Determinations were made of the solubility of zinc arsenite in tap water, distilled water and dilute carbonated water. One series of analyses was conducted according to the official method employed to determine the water-soluble arsenic of paris green, which is done by shaking one gram of the sample in one thousand cubic centimeters of water eight times a day for ten days. The second series of analyses was similar to the above except that the shaking continued for a shorter time. The results are shown in the following table:

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[1] The arsenious and arsenic oxides are not entirely comparable as they are different compounds and probably do not have the same toxic values. It would appear from the experiments described later that as an insect poison .40 lb. arsenious oxide was roughly equivalent to .53 lb. arsenic oxide. There are two other facts about arsenites in general that should be taken into consideration in view of the variable results in the use of zinc arsenite secured by different workers: (1) Arsenites in general are not so stable as the arsenates; and (2) all arsenites usually retain some arsenious oxide so that it is difficult to obtain definite compounds.

Through the courtesy of Dr. L. L. Van Slyke and Mr. E. L. Baker, the moisture determinations of the poisons were made by Mr. Otto McCreary and the analyses by Mr. A. W. Clark.

Solubility of Zinc Arsenite as Indicated by the Water-Soluble Arsenious Oxide.

	Shaken ten days.	Shaken one day.	Shaken one hour.
	Per ct.	*Per ct.*	*Per ct.*
Tap water	.50	.....	.50
Distilled water	.75	.....	.62
Carbonated water	.....	9.67	8.16

The microscopic appearance of the particular lots of the two poisons used in these experiments was as follows: The particles of lead arsenate paste were non-crystalline, roughly spherical and ranged in size from one to three microns. The particles of zinc arsenite were slightly elongated, the shortest diameter being about the same as that of the arsenate of lead.

The materials employed in these tests were used in the following quantities in fifty gallons of the diluted spray: lime-sulphur one and one-fourth gallons of a solution testing 33° Beaumé, bordeaux mixture containing four pounds of copper sulphate and four pounds of quicklime, soap two pounds, furniture glue one pound, glucose twenty-two pounds and quicklime three pounds.

The precipitation record at Geneva, N. Y., covering the period of July 30 to August 24, when tests were conducted in the field, is as follows:

Date.	Rainfall in inches.	
July 31	.06	
Aug. 2	.07	
" 3	.03	
" 5	.08	Total to Aug. 7, .24 inches.
" 10	.44	
" 12	.08	
" 15	.07	
" 18	.65	
" 19	.24	
" 20	.04	
" 21	.03	
" 23	.09	Total to Aug. 24, 1.88 inches.

LABORATORY FEEDING TESTS.

Test No. 1, with Lina scripta.— This test was designed to determine the effects of zinc arsenite and arsenate of lead with and without molasses. The beetles were confined in cages and supplied with sprayed willow foliage. The observations were continued for nine days.

Results.— There was no marked difference in the speed of action of the different combinations with the exception of the zinc arsenite and molasses. With this preparation 60 per ct. of the beetles succumbed within twenty-four hours, while with the other mixtures no insects died until the second or third day. After this period usually one or two of each lot would be found dead every day. A moderate amount of feeding occurred during the first twenty-four hours, then the beetles ceased to feed and remained inactive, as if hibernating, until death ensued.

Test No. 2, with Mamestra picta.— In this test, the purpose was to determine the effects of zinc arsenite and lead arsenate alone or in combination with either sugar, soap or glue. As the insects had been taken on cabbage, seedlings of this plant were used as food. The observations were continued for four days.

Results.— No appreciable difference was detected in the rate of action of the arsenicals on this species. It should be mentioned that neither sugar, soap nor glue influenced materially the effectiveness of the poisons. The toxic properties of the zinc and sugar combination were shown in twenty-four hours by the death of one larva, while the poisonous effects of the other mixtures were not apparent until forty hours. Notwithstanding these differences at the start, all the combinations were practically of equal effectiveness at the expiration of four days.

Test No. 3, with Hyphantria cunea, was made to determine the comparative values of the following combinations: lead arsenate, paste, with water, soap, glucose and glue; lead arsenate, dry, with water, bordeaux and lime-sulphur; and zinc arsenite with water, bordeaux, lime-sulphur, soap, glucose and glue. Trees in the nursery were sprayed with these mixtures and on the same day some foliage of each was brought to the laboratory and fed to the caterpillars. The rate of action of the different mixtures and the relative consumption of food is indicated in Table I.

Results.— Attention is called in this experiment to the similarity in action of the poisons and the irregular amount and character of feeding. However, considering both the appearance of the caterpillars at the end of the test and the number killed, there is a slight difference in favor of the zinc combinations. The maximum feeding occurred with zinc arsenite, zinc arsenite and glue, and lead arsenate and glucose. The minimum amount of foliage was consumed where mixtures of lime-sulphur or bordeaux with either poison had been applied. These results indicate a greater repellent action by the last-named combinations. This repellent property was apparent also in the manner of feeding, for check larvæ fed from the upper surface, leaving the lower epidermis intact; while, as will be noted in the table, the larvæ in some experiments fed only from the under surface or ate holes in the leaf, consuming both the upper and lower epidermis.

Test No. 4, with Hyphantria cunea, was designed to determine the insecticidal values of mixtures after three days' exposure to weather. The sprays applied were lead arsenate, paste, with water, bordeaux, lime-sulphur, soap and glucose; and zinc arsenite with water, bordeaux, lime-sulphur, soap, glucose and lime. Trees in the orchard were sprayed on Aug. 14 and on Aug. 17 foliage was removed from the trees and fed to insects. The rate of action of the mixtures and the extent of feeding are shown in Table II.

Results.— There was very little difference in the rate of action of the poisons except when arsenate of lead was used in combination with lime-sulphur or glucose. These proved less effective, although the amount of foliage actually consumed was greater than with the other lots. This suggests that during the interval of three days' exposure the mixture of lead and lime-sulphur had lost some of its effective properties. The minimum feeding occurred when arsenate of lead was used alone or with bordeaux, and when zinc arsenite was used with lime or bordeaux mixture.

Test No. 5, with Hyphantria cunea.— This test is identical with No. 3 except that the sprayed trees were subjected to the weather for eight days. The trees were sprayed July 30 and the foliage fed to the insects on Aug. 7. As will be seen from the weather record, page 6, there occurred in this interval four showers aggregating, 24 inch.

Results.— All the sprays were still effective as insecticides, though the glucose with lead arsenate had lost some of its poisonous properties. In the eight days' exposure there was a partial loss of the repellent action of some sprays and generally much more feeding than in test No. 3. The minimum feeding occurred with lead arsenate and glue and with the combinations in which soap and bordeaux were used. The area of leaf surface consumed was at least three times greater with mixtures containing lime-sulphur than with mixtures containing bordeaux, which is a marked contrast to what occurred in Test 3. This indicates, as did other tests, that lime-sulphur loses its repellent properties sooner than the sprays that contain soap and bordeaux mixture. The maximum feeding occurred where either lead arsenate or zinc arsenite was used alone or in combination with glucose.

Test No. 6, with Hyphantria cunea.— This is similar to Nos. 3 and 5 except that the sprayed foliage used in this feeding test had been exposed to the weather for twenty-five days. The trees were sprayed July 30 and the foliage brought to the laboratory Aug. 24. The sprayed foliage used in this experiment had been subjected to the washing action of 1.88 inches of rain from twelve showers occurring during the interval.

Results.— The combinations that were still distinctly effective were those in which glue and bordeaux were used and the zinc arsenite and soap mixture. On the contrary, zinc arsenite, lead arsenate,

zinc arsenite with lime-sulphur and the combinations with glucose lost their effectiveness. The precipitation had washed off the evidence of spray from the foliage in all except the bordeaux treatments, which was correlated with more extensive feeding by the insects. This feeding was most abundant where glucose, lime-sulphur and lead arsenate were used, while the minimum feeding occurred with the bordeaux combinations and with the glue and lead arsenate and the soap and zinc arsenite.

FIELD FEEDING TESTS.

Some tests were made in the field to compare the effectiveness of zinc arsenite and arsenate of lead for the spiny elm caterpiller (*Vanessa antiopa*) and the larvæ of the willow beetle (*Lina scripta*).

Results.— The shape and size of the trees upon which the beetle larvæ were feeding prevented actual counts of the insects, but as near as could be determined the time required for effectiveness was practically the same for the two poisons, there being a lapse of four days before all the larvæ died. Opposed to this the results with the *Vanessa* larvæ were quite striking. The spiny elm caterpillars on the trees sprayed with zinc arsenite were all dead within twenty-six hours, at which time the insects on the tree sprayed with arsenate of lead showed no outward effects of the poison, though all succumbed in a period of four days. The experiments with the *Vanessa* larvæ were repeated in the field and in the laboratory with similar results.

Two explanations are suggested to account for this difference in the action of the poisons: *First*, that the zinc arsenite may be more soluble than the lead arsenate in the digestive secretions of these particular insects and that more time is required for the lead than the zinc poison to exert its toxic properties in the body. *Second*, that in drinking from the globules of spray as larvæ were observed to do, more of the zinc on account of its lower specific gravity [1] is actually conveyed into the digestive tract of the insects.

DISCUSSION OF RESULTS.

EFFECTS OF THE POISONS ON THE INSECTS.

In the experiments in which the treated twigs were fed to the insects as soon as the spray on the foliage had thoroughly dried, the effects of the two poisons were generally similar, although there was a slight difference in favor of the combinations containing zinc arsenite, which were quicker in action. The only marked difference between the two arsenicals as regards speed of poisoning was noted

[1] Mont. Exp. Sta. Bul. No. 86.

TABLE I.— TESTS OF KILLING EFFECT OF LEAD ARSENATE AND ZINC ARSENITE ON *Hyphantria cunea* ON APPLE FOLIAGE, GENEVA.

Trees sprayed July 30 and foliage brought into laboratory on same day. Ten larvæ in each test. U = upper surface of leaf; L = lower surface; W = entire thickness of leaf; T = slight injury.

DATE OF OBSERVATIONS.	PASTE LEAD ARSENATE IN COMBINATION WITH —				DRY LEAD ARSENATE IN COMBINATION WITH—			DRY ZINC ARSENITE IN COMBINATION WITH —					
	Water.	Soap.	Glucose.	Glue.	Water.	Bordeaux.	Lime-sulphur.	Water.	Bordeaux.	Lime-sulphur.	Soap.	Glucose.	Glue.
Aug. 2 Number dead	0	0	0	0	0	0	0	0	0	0	1	1	0
Aug. 2 Foliage eaten, sq. cm.	2 U 2 W	5 U	12 U	5 U	½ U ½ L 5 W	3 U	1½ U	10 U	3 L	2 U	4 U ¾ W	3 c. m. W	10 U 10 W
Aug. 3 Number dead	0	0	0	0	0	0	0	0	2	0	0	0	0
Aug. 3 Foliage eaten, sq. cm.	2 U	1 cm.	10 U	2 U	1 W	T	—	10 U	1 U 1 L	T	1 U	T	T
Aug. 4 Number dead	0	0	0	0	0	0	0	0	0	0	1	1	0
Aug. 4 Foliage eaten.	T	T	4 U	T	T	—	—	T	—	—	T	T	—
Aug. 5 Number dead	0	2	0	0	0	0	3	0	0	1	0	1	0
Aug. 5 Foliage eaten.	T	—	T	T	—	—	T	—	—	—	—	—	—
Aug. 6 Number dead	1	2	2	2	0	1	0	0	0	0	0	1	1
Aug. 6 Foliage eaten.	T	—	—	—	T	—	T	T	—	—	—	—	—
Aug. 8 Number dead	5	5	5	5	8	4	2	6	7	8	2	4	6
Aug. 8 Foliage eaten.	—	—	—	—	—	—	—	—	—	—	—	—	—
No. alive at end of test	4	*0	3	3	2	†3	*4	4	1	1	*5	2	3
Total area of leaf eaten	6 U 2 W	6 U 1 W	26 U	7 U 1 L	7 W ½ U ½ L	4 U 1½ W	2 U 1½ W	20 U 4 W	1 U 4 L	3½ U	6 W	4 cm.	10 U 10 W

* One larva parasitized. † Two larvæ parasitized.

TABLE II.— TESTS OF KILLING EFFECT OF LEAD ARSENATE AND ZINC ARSENITE ON *Hyphantria cunea* ON APPLE FOLIAGE SUBJECT TO THE WEATHER.

Trees sprayed Aug. 14 and foliage brought into the laboratory Aug. 17. Twelve larvæ in each test. T = small amount of leaf eaten; M = much injury to leaf; A = whole leaf eaten.

Date of Observation.		Paste Arsenate of Lead in Combination with —					Dry Zinc Arsenite in Combination with —						Check.
		Water.	Bordeaux.	Lime-sulphur.	Soap.	Glucose 22–50.	Water.	Bordeaux.	Lime-sulphur.	Soap.	Glucose, 22–50.	Lime.	
Aug. 20	Number dead	0	1	1	1	1	0	1	0	0	1	1	0
	Sq. cm. of leaf consumed	4 cm.	3 cm.	16 cm.	10 cm.	17 cm.	7 cm.	5 cm.	11 cm.	7 cm.	10 cm.	4 cm.	25 cm.
Aug. 22	Number dead	3	3	3	2	1	1	1	2	1	4	2	0
	Sq. cm. of leaf eaten	T	—	T	T	T	T	T	T	T	—	T	M
Aug. 24	Number dead	0	4	0	2	0	0	4	1	1	2	0	0
	Sq. cm. of leaf consumed	T	—	T	T	T	T	—	—	—	—	—	A
Aug. 26	Number dead	5	3	2	7	4	9	5	8	9	5	9	0
	Sq. cm. of leaf consumed	—	—	T	—	—	—	—	—	—	—	—	—
Alive at end of test		*3	1	*5	0	6	2	*0	1	1	0	0	12
Total feeding		4 cm.	4 cm.	16 cm.	11 cm.	20 cm.	7 cm.	6 cm.	12 cm.	7 cm.	10 cm.	4 cm.	—

* One larva parasitized

when caterpillars were on the foliage at the time of treatment or placed on the tree when the leaves were still wet with the spray mixture. In this case applications of zinc arsenite showed their deadly effects upon the caterpillars much sooner than those containing lead arsenate.

As a rule the insects on freshly-sprayed foliage ate only one meal and thereafter fed very little until death ensued or the experiment was concluded. In all cases the amount consumed in the initial meal was greater with the check than with any of the sprayed plants, indicating that either there was a reaction by the insect to the poison or that the spray possessed distasteful or repellent properties. In the tests in which foliage that had been exposed to the weather was fed to insects the period of feeding was more extended, depending on the interval between treatment and the feeding of the insects. The caterpillars which were given foliage that had been exposed for twenty-five days fed voraciously for three to six days before the poison became effective.[1]

EFFECT OF WEATHERING ON EFFICIENCY OF SPRAYS.

The effects of weather on the insecticidal properties of applications of the poisons are shown in Tests 3, 4, 5 and 6. The period of exposure of the sprayed foliage was three hours, three days, eight days and twenty-five days respectively. See Chart I, (p. 16).

All sprays were practically equal in effectiveness when first applied, but exposure to weather tended to reduce their insecticidal properties. In these experiments the preparations that contained bordeaux, soap or glue in combination with either of the poisons and the lime-sulphur combined with dry lead arsenate continued effective as insecticides for twenty-five days. Zinc arsenite or arsenate of lead alone or with glucose ceased to protect the foliage from insects at the conclusion of this period.

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[1] The effect of arsenic upon the higher animals and man depends upon the size and form of the dose administered and the condition of the subject. According to Blyth (Poisons: Effects and Detection, pp. 494–543. 1884) a large dose results in an acute case. The symptoms usually appear within an hour and may be accompanied by vomiting and diarrhœa, death following in from 3 to 24 hours. The symptoms resulting from a milder dose are much the same, though death may not occur for a longer period. Where the dose is very mild the ordinary vomiting and purging are much suppressed and instead jaundice, narcotic sleep or partial paralysis may result. These symptoms may be followed by death or the subject may recover after an illness varying from a few days to several months. The behavior of poisoned animals corresponds very well with that of the insects under observation. The caterpillars which were supposed to have received a large dose were affected with a severe diarrhœa and died within 20 to 30 hours. Some individuals were apparently affected more than others, judging from the interval between feeding and death. In other tests the insects became inactive, some ceased to feed, others would nibble here and there until death ensued; while many of the larvæ after one meal of poisoned foliage showed symptoms of starvation, becoming less active, with an apparent drying of the body.

All of the sprays were distinctly repellent at first. On exposure to weather some sprays lost their distasteful or repellent properties much sooner than others. The mixtures containing bordeaux, soap and glue were repellent to the end of the test, covering a period of twenty-five days. As opposed to this the poisons when used alone or with lime-sulphur lost their repellent properties by the end of the period. The loss of the distastefulness of the mixtures that contained lime-sulphur is to be noted as they were the most repellent at the initiation of the test. Lead arsenate and glucose, which was the least repellent of all the combinations used, soon lost its insecticidal properties when the foliage was subjected to the weather.

SAFENESS OF ZINC ARSENITE FOR USE ON FOLIAGE.

The purpose of these tests was to determine the safeness of zinc arsenite for use on foliage and its usefulness when combined with other sprays. Apple, pear, plum and peach trees in the Station nursery and individual trees of the dwarf orchard were used. Zinc arsenite was applied to five acres of grapes in the region of Fredonia by Mr. F. Z. Hartzell of the Chautauqua Grape Laboratory of the Station. Applications to elm and willow were made in a local nursery and several sprayings were made in a large basket-willow plantation at Lyons, N. Y.

EFFECT ON APPLE FOLIAGE.

EFFECTS ON APPLE FOLIAGE OF LEAD ARSENATE AND ZINC ARSENITE ALONE AND IN COMBINATION WITH OTHER MATERIALS.

Lead arsenate, paste	Water	No injury.
" "	Lime-sulphur	No injury.
" "	Bordeaux	No injury.
" "	Soap	No injury.
" "	Glucose	Slight marginal burning.
Zinc arsenite, dry	Water	Slight marginal injury.
" "	Lime-sulphur	Very slight spotting of foliage.
" "	Bordeaux	No injury to healthy foliage.
" "	Soap	Injury slight, confined to margins and points of injury.
" "	Glucose	Injury moderate to severe.
" "	Lime	No injury.
Lead arsenate, dry	Water	No injury.
" "	Lime-sulphur	No injury except to broken leaves.
" "	Bordeaux	No injury.

It is to be noted that no injury to apple foliage resulted from the use of arsenate of lead alone or in combination with bordeaux or lime-sulphur, or from zinc arsenite with lime or bordeaux. Zinc arsenite alone or in combination with soap, glucose or lime-sulphur caused more or less injury. Zinc arsenite caused spotting of the leaves and injury about the margins. The spotting did not appear

until several days after treatment but was evident in twelve days, at which time the worst injured leaves had turned yellow. With zinc arsenite and lime-sulphur the same type of burning was present but to a less degree while in combination with glucose the injury was severe.

EFFECT ON GRAPE FOLIAGE.*

Test No 1.— On June 22 ten vines were sprayed with zinc arsenite at the rate of six pounds of the poison to one hundred gallons of water. By July 26 there was severe burning of foliage and fruit.

Test No. 2.— On June 24 ten vines were sprayed with six pounds of arsenite of zinc and two gallons of molasses in one hundred gallons of water. Foliage and fruit showed severe burning by July 26.

Test No. 3.— On July 12 one acre of grapes was sprayed, using zinc arsenite two pounds, molasses two gallons and water one hundred gallons. In two weeks injury was shown similar to that in the preceding tests.

Test No. 4.— On July 13 one acre of grapes was sprayed using two pounds of zinc arsenite to one hundred gallons of bordeaux mixture (formula 8–8–100). No injury resulted.

Test No. 5.— One acre of grapes was sprayed July 15 using two pounds of zinc arsenite to one hundred gallons of water. Injury was apparent within a week.

Test No. 6.— On August 16 a vine was sprayed with zinc arsenite two pounds, lime one-half pound and water one hundred gallons. No injury resulted.

The weather during the period of June 22 to July 12, the dates of the preliminary tests and the field experiment, was dry and hot, but after July 15 there was considerable rain and cool weather; so that the results noted above indicate that wet weather favors injury to the foliage by this poison. From June 22 to July 14 the average mean temperature was 73.1°, with a maximum of 91° but no rain. From July 15 to 25 the average mean temperature was 66 with a maximum of 83°. Rain fell on the 15th, 18th, 21st, 24th and 25th; 1.33 inches falling during this time.

Results.— Zinc arsenite when used alone or in combination with molasses caused severe burning of fruit and foliage of the grape. In these tests the injury was not apparent for a period varying from one to three weeks. Zinc arsenite when used in combination with lime or bordeaux mixture caused no injury to the foliage.

OTHER TESTS OF ZINC ARSENITE ON FOLIAGE.

In a few other preliminary experiments the results are as follows: A slight marginal injury occurred to the foliage of pear and plum. Peach leaves were severely scorched. Zinc arsenite caused no

* These tests were made and reported by F. Z. Hartzell, Associate Entomologist.

injury to the foliage of elms or to potatoes and cabbage. Willow leaves were only slightly spotted.

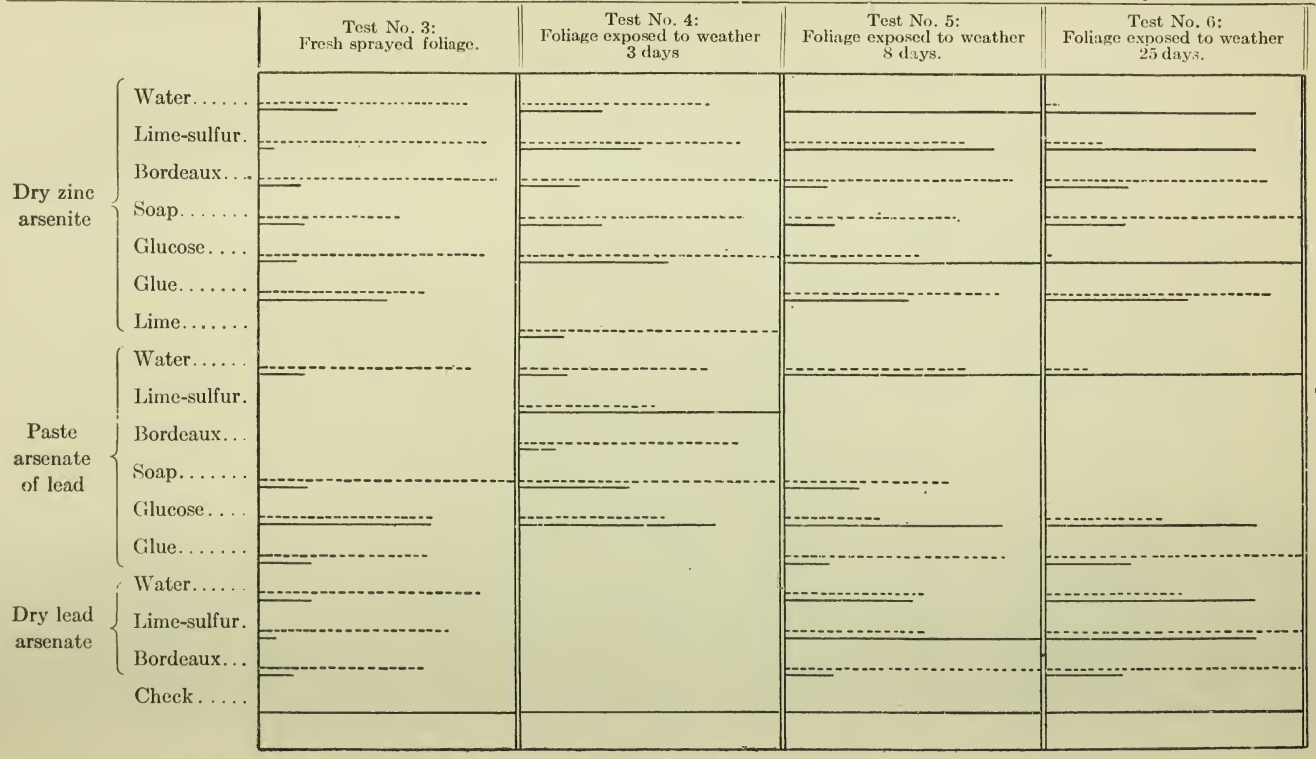
CONDITIONS THAT ACCOMPANY THE INJURY TO APPLE AND GRAPE FOLIAGE.

Attention is called to several of the above mentioned observations regarding the injury to foliage. The burning was prevented by lime and was increased by the addition of molasses. It did not occur immediately following the application of the poison, but only after several days; and then apparently it was favored by wet weather. As noted by Hartzell in the experiments on grape foliage, zinc arsenite when combined with lime or bordeaux mixture caused no injury. The same is true in the tests on apple foliage. The lime would function as an absorbent of carbon dioxide and would also unite with any soluble arsenic present, forming calcium arsenite.

The occurrence of the injury during wet weather points to leaching of the arsenic as a contributory cause. But the low water-solubility of zinc arsenite, namely, one per ct. at a dilution of one to one thousand by weight, together with the fact that several days are required before the injury occurs, would indicate the necessity of some solvent other than water or that the compound is altered chemically before the burning takes place. The only suggestion in these tests as to the cause for the injury to the apple and grape foliage by zinc arsenite is the ease with which the material enters into solution in dilute carbonic acid. It is generally assumed that due to the process of respiration, carbonic acid is present at times in small amounts on the leaf surface. It is possible that the combined action of the wet weather and the solvent properties of the carbonic acid are partly responsible for the damage.

Chart I.—Efficiency of Poisons Exposed to Weather.

——— Relative feeding of caterpillars as compared with check.
..... Effectiveness as determined by the percentage of caterpillars killed and condition of those alive at the termination of the experiment.



Note.—In experiments 3, 4 and 5 the length of the black line is based on the number of sq. cm. of estimated feeding that occurred during the first part of the experiment. In test 6 the feeding was generally such that it was not feasible to estimate the area and instead the expressions extensive, moderate, etc., were used. Upon these the line is based. Dotted line omitted in first section under Test 5 because caterpillars escaped after eating foliage.

TECHNICAL BULLETIN No. 29. MARCH, 1913.

New York Agricultural Experiment Station.

GENEVA, N. Y.

AN EFFICIENT ELECTRICAL INCUBATOR.

H. JOEL CONN AND H. A. HARDING.

PUBLISHED BY THE DEPARTMENT OF AGRICULTURE.

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*Riverhead, N. Y. †Absent on leave. ‡Connected with the Chautauqua Grape Work.

TECHNICAL BULLETIN No. 29.

AN EFFICIENT ELECTRICAL INCUBATOR.

H. JOEL CONN AND H. A. HARDING.

SUMMARY.

1. The ordinary form of gas-heated incubator is unsatisfactory because of the danger of fire and the difficulty in securing constant temperatures below that of the room.

2. The difficulty has been avoided by constructing incubators insulated with cork-board, heated by electricity, and one of them cooled by the drip-water from a refrigerator.

3. The cost of constructing four incubators, each of 7 cu. ft. capacity, and a refrigerator of 25 cu. ft. capacity, was $382.

4. The cost of maintenance, with the incubators at 18°, 25°, 30°, and 37° C. respectively, and the refrigerator at 7° to 10° C., is well under $40 per year.

INTRODUCTION.

One of the necessities for careful bacteriological work is a means of incubating cultures at various temperatures. Temperatures above that of the room are easily secured, but satisfactory incubators which remain constant at lower temperatures are almost unknown. For this reason it seems worth while to describe a piece of apparatus providing for incubation at both high and low temperatures, which has been used here for a year with entire satisfaction. It consists of a refrigerator and four incubators, and is heated by electricity.

CONSTRUCTION OF REFRIGERATOR AND INCUBATORS.

MATERIALS.

In constructing the refrigerator and incubators the following materials were employed.

Lumber. The outer case was built of matched, seven-eighths-inch, kiln-dried oak. In order to necessitate as few battens as possible, and to furnish a smooth inner surface on which to lay the succeeding layers of insulating material, strips of this oak were glued together edge to edge, alternating the grain so as to prevent warping. Thus each side of the case is essentially a single board. To protect the wood from the action of dampness the inner surface was finished with three coats of paint, while the outer was filled and varnished.

Well seasoned white pine was employed for an inner lining of the refrigerator. This was given three coats of paint on each side and both tongue and groove were freshly painted just before laying.

Posts made of seven-eighths-inch whitewood glued up to prevent warping were used for door-jambs and for the framework of the doors. Paint was used to waterproof these posts on all surfaces except those edges where the doors and jambs were to come in contact. As neither paint nor varnish could be used there, and as oil alone would cause the wood to swell, these edges were treated with paraffin. The paraffin was driven into the wood by means of a hot iron. The result of this treatment, in protecting from moisture and in preventing the door and jamb from sticking together, has been entirely satisfactory.

Half-inch seasoned cherry was used for the incubator shelves and their supports, and seven-eighths-inch seasoned oak for the ice-rack and ice-fenders in the refrigerator. Since these were to be exposed to considerable moisture, they were thoroughly impregnated by stewing the finished pieces for at least thirty minutes in melted paraffin. The oak ice-rack, after this treatment, although it stands constantly in water, has not warped in the slightest; but in the shelves of the 18° incubator, which contains a saturated atmosphere, some warping has occurred.

Throughout the construction, every piece of lumber was treated over its entire surface with paint or other substance designed to prevent the entrance of moisture.

Insulating material.— Cork-board [1] was the main provision for insulation. This is a very efficient insulating material, and since it can be cut accurately by either saw or bit, lends itself readily to close construction. The sheets of cork-board used were four inches thick, and one foot wide and three feet long. A single layer of this thickness has proved satisfactory; but two layers of three-inch stuff, laid with broken joints, would undoubtedly have furnished better insulation, thus reducing both the consumption of current and the heat transference from chamber to chamber. The joints between the sheets of cork were cemented with Nonpareil Waterproof Cement.[2] This cement has served its purpose perfectly; but it is so expensive that some other binding substance might prove cheaper and more satisfactory.

Metal.— Heavy zinc was used in lining the refrigerator to protect the walls from moisture and from mechanical injury.

Shelves, shelf supports, and drip-pan, in the refrigerator, were made of galvanized iron.

Brass pipe was used for the drain to the refrigerator and the cooler of the 18° chamber. Its external diameter is at most places one inch, but a short length is of inch-and-a-quarter pipe. The use

[1] Nonpareil Corkboard, obtained of Armstrong Cork Co., Pittsburg, Pa.

[2] Obtained of Armstrong Cork Co.

of brass is to keep down fungus growth within the pipe, not so much because of the toxic action of the brass itself, as because copper sulphate can be added at intervals without causing electrolytic action.

In each of the incubators steel bars were used to support the upper layers of cork. Strips of wood impregnated with paraffin, however, having lower conductivity to heat, would undoubtedly have caused less heat transference from chamber to chamber.

Hinges and door clasps were of brass.

Miscellaneous materials.— Between the oak casing and the cork-board of both refrigerator and incubators was laid the best obtainable quality of building paper.

In the incubators a Nonpareil Finishing Cement [3] was applied directly to the inner surface of the cork. The cement was covered with three coats of paint to secure a harder and more waterproof surface. This cement was used, instead of ordinary Portland cement, because it was understood to be less liable to crack. In the 37° incubator, however, cracks have formed; and as this cement sets slowly and never becomes very hard, its use is hardly to be recommended.

CONSTRUCTION.

The dimensions and main details of construction of the refrigerator and incubators are shown in Figs. 1–3. Fig. 1 is a front elevation, while Figs. 2 and 3 are sections to show the construction in detail. The present description is merely supplementary to these figures.

Construction of refrigerator.— The refrigerator is 35x40x81 inches over all and has a capacity of 25 cu. ft. These dimensions are not ideal, but were determined by the space available. Had space permitted, a broader ice-chamber, which could better accommodate an ordinary cake of ice, would have been more satisfactory.

In order to secure rigidity the outer case of oak was built with box headings at the top and bottom, as shown in Fig. 2, carefully breaking all the joints and laying the battens in fresh paint. Inside these headings, after building paper had been tacked over the oak, the four-inch layer of cork was fitted snugly into place. The headings were then covered with matched pine. The building paper and cork-board were next fitted closely around the back and sides. This layer of cork was covered in turn with seven-eighths-inch white pine. The resulting box, 23x28x69 inches, was lined with heavy zinc, the angles flushed with solder, and the front edge of the zinc nailed closely to the front edge of the pine lining.

To support the drip-pan and the ice-rack which lies in it, a wooden shelf was built 20 inches from the top of the chamber. In front

[3] Nonpareil High Pressure Finishing Cement, obtained of Armstrong Cork Co.

and in back of this shelf a two-inch space was left to permit circulation of air.

The galvanized iron drip-pan has a brass outlet secured by gaskets and lock-nuts in such a manner as to project about an inch above the bottom of the pan. This outlet extends less than an inch below the pan, and slips snugly into the brass pipe which conducts the water through the wall of the refrigerator into the

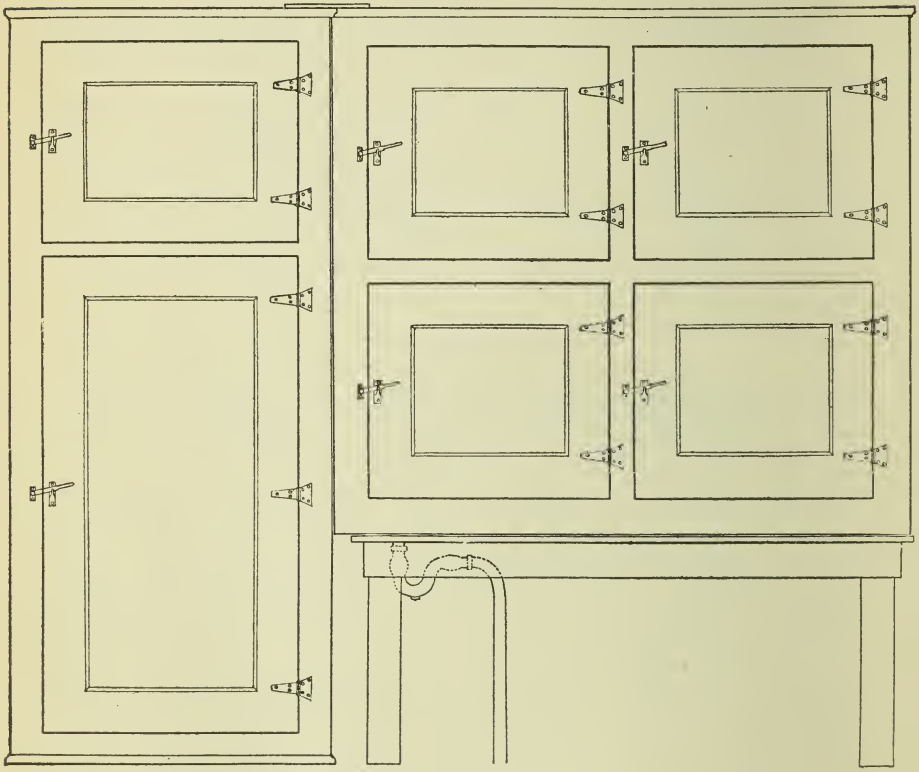

FIG. 1.—FRONT ELEVATION OF REFRIGERATOR AND INCUBATOR.

incubator. The shortness of the outlet and its slip-joint allow the pan to be easily removed for cleaning or inspection; while the protrusion of the outlet pipe above the bottom converts the drip-pan into a sedimenting chamber to remove the solids derived from the ice. This arrangement is to protect the drainage system from clogging and to facilitate cleaning in case clogging should occur.

From Figs. 1 and 3 it can be seen that the front of the refrigerator consists wholly of doors and door-jambs. The outer layer of the doors is of oak, glued up with a panel effect on the outside, but smooth and flush on the inner surface. The edges of the doors and the jambs are beveled on all sides in order to secure a close fit. The central portion, making up about two-thirds of the entire door,

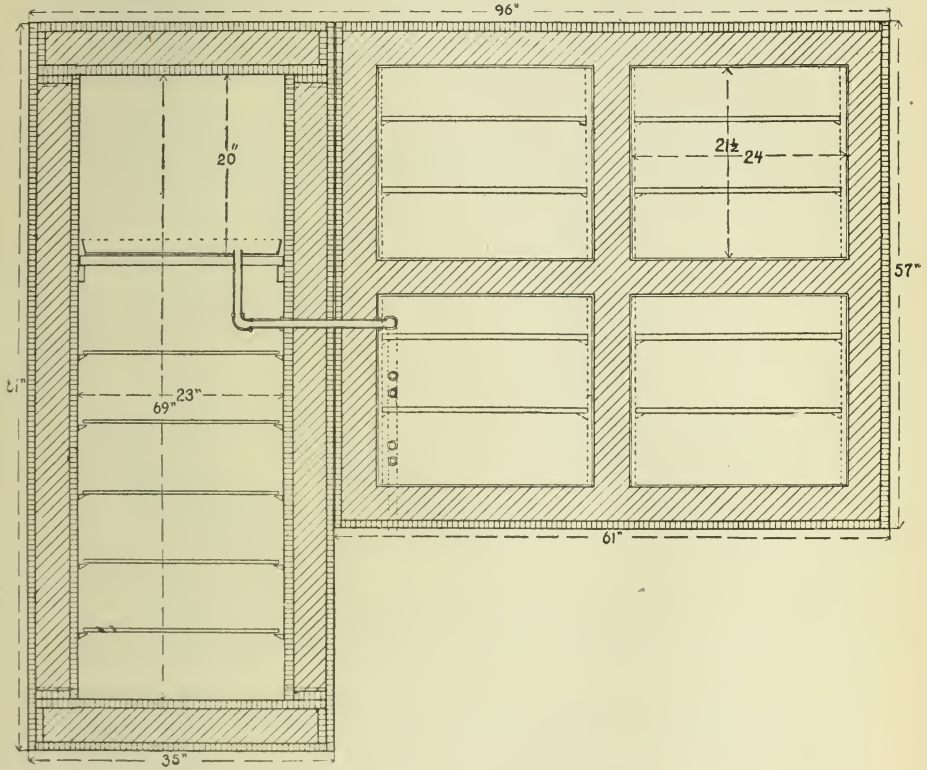

FIG. 2.—VERTICAL SECTION OF REFRIGERATOR AND INCUBATOR.

(Diagonal hatching indicates cork-board; other hatching, wood.)

is a layer of four-inch cork-board, the inner surface of which is protected by a sheet of zinc nailed securely to the whitewood edges.

The upper door is for admitting ice. The inner edge of the threshold is covered with a strip of galvanized iron and is slightly lower than the top of the ice-rack. Thanks to this device a large cake of ice, raised by rope and pulley, can be swung into place without

damaging the threshold. As a further protection from the ice, the walls and inside of the door of the ice-chamber are provided with fenders of oak impregnated with paraffin. These fenders, moreover, prevent the ice from slipping out of place, so that all the water from its melting falls into the drip-pan. The ice-chamber readily accommodates 200 pounds of ice.

The lower door opens into the storage chamber. Just inside of this door two galvanized iron doors are hung one above the other. One of these is shown in section in Fig. 3. They are designed to minimize the loss of cold air when access is desired to the upper shelves of the storage chamber. The storage chamber contains

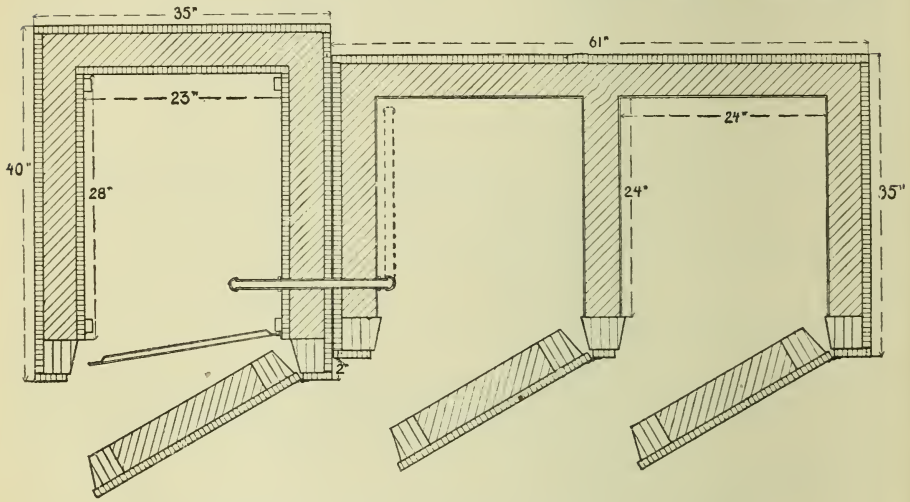

Fig. 3.—Horizontal Section of Refrigerator and Incubator.
(Diagonal hatching indicates cork-board; other hatching, wood.)

approximately 17½ cu. ft. The five shelves within it, as shown in Fig. 2, are supported by runners of angle iron and are easily removed. This arrangement of shelves affords about 27 sq. ft. of shelf space. One of the shelves is designed for cooling gelatin plates.

Construction of incubators.— As shown in Figs. 1–3, the four incubators, each of 7 cu. ft. capacity, are built together as a single unit. This arrangement, as well as the shape of the chambers, was made necessary by the space available; but as a result the walls are so thin as to allow some heat transference from incubator to incubator, and the chambers so high as to bring about a difference of one or two degrees in temperature between top and bottom. Had each chamber been built separately, the insulation would have been more complete and the incubators, at the same time, would have

been portable; while if they had been broader and lower (15 inches high, possibly) with one shelf instead of two, the temperature variation between top and botton could probably have been disregarded.

The details of construction are similar to those of the refrigerator except for the omission of pine lining and of the accessories for supporting the ice.

The walls separating the individual incubators consist solely of four-inch cork-board, cemented together, the horizontal partitions having, on their under surface, two thin bars of steel for support. The construction is further strengthened by a steel rod passing through the cork-board from the middle of the front to the middle of the back, serving to prevent the front and back from spreading. The inner surfaces of the cork are covered with the Nonpareil finishing cement, except on the doors, where zinc is used; and both cement and zinc are painted white. In each chamber there is a cherry framework around the walls to support shelves and thermostat. The two shelves are built of slats of half-inch seasoned cherry and rest on metal pegs so that they can be adjusted to any height. A fender about an inch in front of the thermostat, which stands at the middle of the back wall, protects it from injury.

HEATING SYSTEM.

The arrangement of the circuits in the incubators is shown diagrammatically in Fig. 4. In each incubator there are but two essential pieces of apparatus, the thermostat T, and the resistance wire R. The four incubators are connected with the main circuit through the fuses FF, and are in parallel, just as are ordinary incandescent lights. Within the chambers, however, the thermostats and heaters are in series.

The thermostat is the most important part of the heating system. The four thermostats were designed and made by Prof. C. H. Tower of the electrical engineering department at Cornell University. They are wholly of metal, with rigid bases, and can be firmly secured in any desired position. The sensitive part of each is a strip of copper and iron about twelve inches long. As this type of thermostat interrupts the current when over-heated and can be wired into the main circuit, power is used only when it is needed for the production of heat. This results in economy. The initial cost, moreover, of all four thermostats was only $12.

The heaters are of Nichrome wire [4] No. 32, having a diameter of 0.008 inch. In each chamber are two lengths of wire, each about 20 feet long, shown in Fig. 4 by the zig-zag lines RR. In the incubators, however, the turns of wire are in a horizontal instead of a vertical plane, and each length is supported beneath a shelf. As shown in Fig. 4, the two lengths of wire are not arranged the same

[4] Obtained of Driver-Harris Wire Co. of Harrison, N. J.

in all four chambers; for temperatures above 30° they are in parallel, but for temperatures as low as 25° they are in series. This arrangement allows four times as much current to pass through the heaters for the higher temperatures as through those for the lower ones. With stationary shelves this arrangement of the wires would have presented no difficulties; but it was desired in this case to have removable shelves. To accomplish this the ends of the heating wires are fastened to copper plates at the edges of the shelves; and when the shelves are in position these plates are in contact

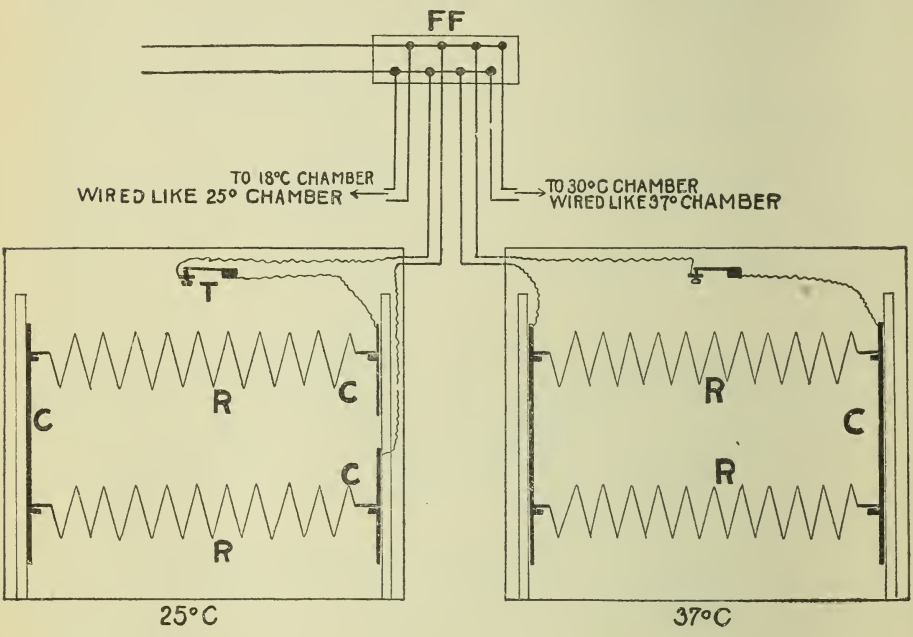

Fig. 4.—Arrangement of Circuits.

T, thermostat; R, resistance wires; C, copper plates; FF, Fuses.

with the metal pegs that support the shelves. In each chamber two of the supporting posts CC are faced with copper; and as this copper is connected by wire with the thermostat and the main circuit, the current passes through the heating wires whenever the thermostat is closed. This arrangement of the heaters places them well out of the way and distributes them so as to prevent overheating at any part of the chamber. To avoid the difficulty of wiring beneath the shelves, the heaters might have been placed vertically at each side of the chamber. This lateral arrangement, although not tried out here, might prove fully as satisfactory.

In placing uninsulated wire so close to paraffined wood, there might seem to be some danger of fire. This danger, however, is more apparent than real. The wire used offers so much resistance that even though the thermostat should fail to break the circuit the temperature would not become extremely high; and if the improbable should happen and a sufficiently high temperature should be reached, the renewal of oxygen through the cracks of the doors would not be sufficient to support combustion. Even though it were, there would be nothing to burn but the inside woodwork, for cork-board is incombustible. As a further protection, however, each length of wire has been broken at one point and soldered together again in such a way that if the solder melts the two ends of wire will fall apart and break the circuit. Using these precautions, there has been no indication of the slightest danger from fire during a whole year of use. Compared to the danger of fire from a gas incubator the fire risk here is wholly negligible.

Each chamber is connected with the main circuit by means of what is called deck cable. This double-wired cable passes through a close fitting hole in the back of each chamber, where it is held in place by means of cement (the same as used for the cork-board), which was smeared upon the cable before drawing it into its final position. Outside the incubators, the only electrical appliance is the fuse-box FF.

All external resistance is avoided; because with a voltage of 110 or 120 sufficient resistance is furnished by the heating wires to prevent the passage of too powerful a current. The advantage of this arrangement is obvious, for it allows practically all the energy to be utilized within the chamber. The avoidance of external resistance and the good heat insulation insure a low cost for maintenance.

COOLING SYSTEM.

There are three principal methods of cooling ordinarily used in low temperature incubators. The first is a circulatory system of either pure water or brine; the second is by means of a continually flowing stream of cold water; while the third method is employed in the Panum type of incubator where cooling is effected by direct conduction from an ice-chamber through thin, uninsulated walls. The first of these three, as it often requires an extra thermostat, is quite complex; and the last method is very inaccurate; while the second, provided a stream of sufficiently cold water can be obtained, is both simple and accurate. Because of the difficulty in obtaining such a stream during hot weather, this method is slightly modified in the present incubator by using as a cooling medium the drip-water from the ice-chamber.

The 18° chamber alone is cooled. The cooling apparatus consists of a one-inch brass pipe through which flows the drip-water

from the refrigerator. The total length of pipe is 11 ft., and it passes four times across one side of the chamber. Fig. 5 is a diagrammatic sketch of the wall of the chamber which bears this cooler. At A is a screw union securing the cooler to the pipe which enters from the refrigerator. At B is a slip-joint where the cooling pipe rests in the drain. C is a stand pipe of one-and-a-quarter-

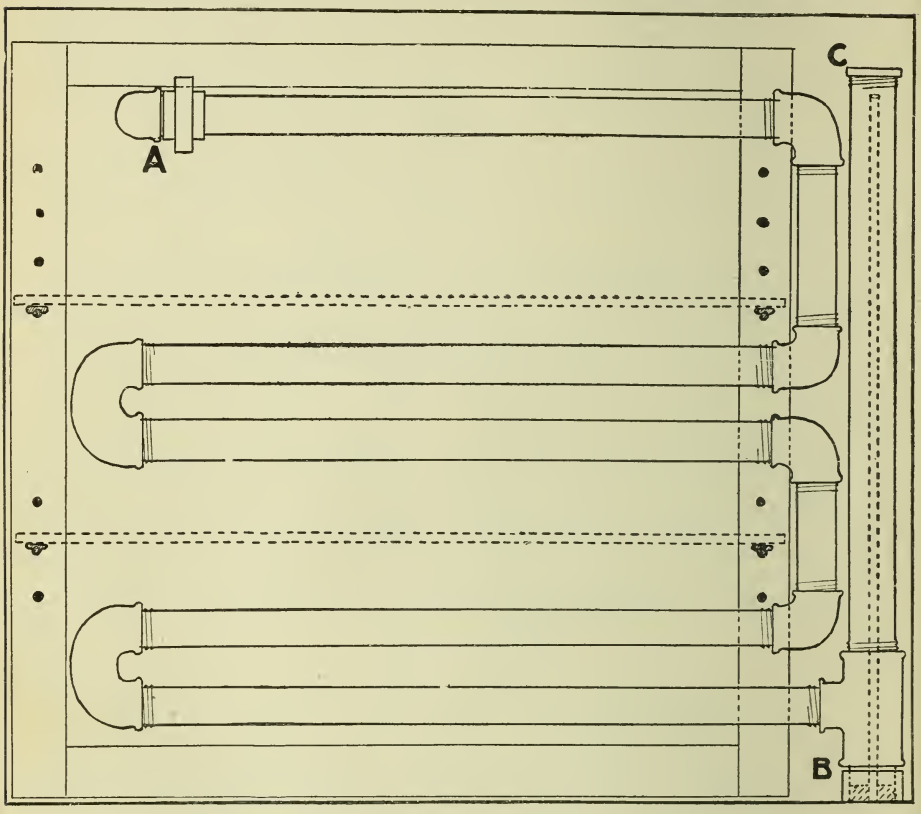

Fig. 5.—Wall of 18° Chamber, Showing Cooler.

A, screw union at entrance from refrigerator; B, slip-joint at exit into drain; C, stand-pipe.

inch brass tubing. Within the stand-pipe is a glass tube passing through a rubber stopper which fits tightly into the outlet at B, thus forcing the water to run out through the tube. By changing the length of this glass tube the amount of cold water retained within the cooler is easily regulated. As the cooler is attached only at the screw union A and the slip joint B, it is readily removed for cleaning.

The drain from this cooling pipe, as shown in Fig. 1, is connected with the main drain of the building through a trap. There is a space of but five inches between the water level of the trap and the point where the pipe leaves the incubator. This is an important point; because the air within the pipe may be warmed for this short distance and carry the heat into the incubator through convection. The longer the distance, therefore, between the trap and the incubator, the greater will be this warming effect upon the chamber.

It has been found in practice that when the glass tube is adjusted to keep the overflow from the cooler almost as high as its entrance into the chamber, and the pipe is full of water, the temperature of the chamber, even without the use of a thermostat, remains fairly constant at 17°–18°. If a temperature of 20° were desired, the outlet from the cooling pipe could be lowered by shortening the glass tube, allowing less cold water to stand inside the chamber. The regularity effected by the cooler is surprising; but it is undoubtedly explained by the fact that the warmer the weather the faster the ice melts and the more cold water is supplied to the chamber. Still greater regularity is secured by the use of thermostat and heater as well as the cooler, which keep the temperature constant at times when the cooler alone would bring it below the desired point.

The cooler keeps the temperature of the incubator below 18° except when the room temperature exceeds 28°. Its failure at such times might have been prevented by building a larger ice chamber or possibly by constructing the cooler with more radiating surface. In this climate, however, these high temperatures are so rare that the arrangement described has given no serious trouble.

The chief objection to using the drain pipe for cooling purposes is its liability to clog with sediment or with the fungus growth that often collects in drains of ice-chests. The sediment, however, is kept out by having the overflow from the drip-pan, as already mentioned, half an inch above its floor; and the fungus growth is largely prevented by putting a little copper sulphate into the pipe at intervals. The trouble with clogging, indeed, has been very slight. During the ten months of service the pipe has been cleaned out but four times; and after the practice was begun of adding the copper sulphate as often as every ten days, four months passed without the necessity of cleaning. The ease, moreover, with which the whole cooling pipe can be disconnected makes it a moderately simple matter to remove it when it does need cleaning.

This form of cooler, indeed, has proved entirely satisfactory. It is more accurate than the Panum type, and more simple than a circulatory system. It also has an advantage over the ordinary incubator cooled by a non-circulating stream of water in that its cooling medium is especially abundant at the season when it is most needed.

COST.

Cost of construction.— If no allowance be made for the time spent by members of the Station staff in supervision, the cost of construction was as follows:

Carpenters (labor)	$134 00	Other electrical fittings	$15 00
Cork-board	45 00	Zinc and galvanized iron fittings (and labor)	34 00
Cement for cork-board	11 50	Hinges, door-clasps, screws, nails, etc	12 00
Lumber	67 90		
Mason (labor and materials)	17 00		
Finish (paint, varnish, etc.)	10 00		
Plumbing	23 00	Total	$381 75
Thermostats (and heating wire)	12 35		

Cost of maintenance.— The incubator has proved especially economical in service. The only cost of maintenance has been for ice and electricity. Ice consumption is very moderate. During the spring, while the room temperature averaged 21°, it was found that 216 lbs. of ice lasted 12 days. Assuming the cost of ice to be $5 per 2,000 lbs., this makes the cost $0.30 per week; and as the week was of about average temperature, it may be concluded that the cost per year is about $15 for ice.

The cost of the current consumed is also moderate. The insulation is so effective that approximately only one watthour a day is consumed per square foot of wall per degree Centigrade difference in temperature outside and inside. To make allowance for the occasional opening of the doors when the incubator is in moderate use, 10 per ct. is a fair amount to add to this, although the heat loss from this cause varies with the temperature of the incubator, temperature of the room, and frequency of opening the doors. These figures, however, are useful only for rough computation, as they were obtained by means of a commercial meter[5] which had not been standardized and was adapted for higher loads than furnished by the heaters. The radiation through cork-board of one inch thickness, as stated by the manufacturing company, is 6.0 British Thermal Units per square foot, per degree Fahrenheit difference in temperature. Based on this estimation, 0.786 watthours a day would be required for four-inch cork-board per square foot per degree Centigrade difference in temperature.

In actual use this has worked out as follows: With an average room temperature of 19° C, and the incubators in constant use at 37°, 30°, 25°, and 18°, respectively, 730 watthours have been consumed per day or about 255 kilowatthours per year. Allowing a cost of $0.10 per kilowatthour, the total cost per year is only $25.50.

[5] Thomson Watthour Meter (2 wire): Volts 100–110: Amp. 5; loaned by the courtesy of the Central New York Gas and Electric Co., Geneva, N. Y.

The total cost of maintenance is thus seen to be about $40 per year if based on the estimates of $5 per ton of ice and 10 cents per kilowatthour of electric power. These estimates are purposely high, and with the rates current here the cost is much less.

EFFICIENCY OF APPARATUS.

The efficiency of an incubator for bacteriological work is principally a matter of accuracy in temperature control. The temperature regularity of this incubator may be seen at a glance from Fig. 6

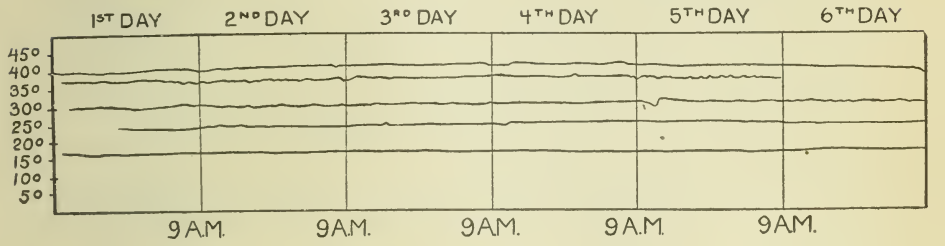

FIG. 6.—THERMOGRAPH RECORDS.
At 17.50°, 25°, 30°, 37° and 40° C.

which contains temperature curves obtained by means of a thermograph in the various chambers at 17.5°, 25°, 30°, 37° and 40°. All the variations are within one degree Centigrade except the one great irregularity in the 30° curve during the fifth day; and this irregularity was the result of shutting off the power for two hours. The most noticeable of all the other irregularities occur early in the morning; and it is thought they must be due in some way to the suddenly increased demand for electric power at the beginning of Station hours. The low temperature curves are particularly regular. This is especially interesting because of the difficulty often experienced in obtaining efficient incubators for temperatures below that of the room.

There is a difference in temperature, fully as great as this, between the top shelf and the floor of each incubator. This difference is 2° for chambers run at 30° or 37°, and 1° for chambers run at 18° or 25°. With chambers as high as these this difference is inevitable; but it can be allowed for by using the same shelf always for the same work.

In the refrigerator no attempt has been made to secure regularity in temperature. It varies with the outside temperature and the amount of ice within, and ranges between 7° and 10° C.

The whole piece of apparatus, indeed, seems to be very efficient, and, considering its size and accuracy, moderately inexpensive.

CONCLUSIONS.

A piece of apparatus built recently for this Station is a combination of four incubators, of 7 cu. ft. capacity each, and a refrigerator of 25 cu. ft. capacity. The incubators furnish temperatures between 18° and 50° C; while in the refrigerator a temperature of from 7° to 10° is obtainable.

The special features of this incubator are: electric heating, with all the power utilized in heat production inside the chambers; cooling by means of the drip-water from the refrigerator; and heat insulation by walls of four-inch cork-board.

This type of construction has the following advantages:

1. It is efficient. Heat control is accurate, and great constancy of temperature can be maintained.
2. It is not expensive. Cork-board is relatively cheap, and the cost of the heating apparatus is under $15. Total cost of construction under $400.
3. Cost of maintenance is low. The thorough insulation of the walls and the utilization of all the current within the chambers prevents loss of heat and therefore minimizes the consumption of power. Total cost is below $40 per year.
4. The risk of fire is negligible. Cork-board is not combustible, the renewal of oxygen through the cracks of the doors is too slow for active combustion, and the solder joints of the heating wires would melt and break the circuit before reaching a dangerously high temperature.

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TECHNICAL BULLETIN No. 30. JULY, 1913.

New York Agricultural Experiment Station.

GENEVA, N. Y.

THE INFLUENCE OF TEMPERATURE AND MOISTURE IN FUMIGATION.

W. J. SCHOENE.

PUBLISHED BY THE DEPARTMENT OF AGRICULTURE.

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*Riverhead, N. Y. †Absent on leave. ‡Connected with the Chautauqua Grape Work. §Elect; on duty after August 1st. **Connected with Hop Culture Investigations.

TECHNICAL BULLETIN No. 30.

THE INFLUENCE OF TEMPERATURE AND MOISTURE IN FUMIGATION.

W. J. SCHOENE.

SUMMARY.

This bulletin discusses a series of fumigation tests with caterpillars of the brown-tail moth (Euproctis chrysorrhœa Linn.), which were influenced by temperature and humidity.

A greater number of caterpillars survived the fumigations made at low temperatures than at higher temperatures; also fumigations made under humid conditions were uniformly more destructive to the larvæ than tests that were conducted in a relatively dry air.

It is suggested that the differences in the results referred to above and the unusual resistance of the caterpillars to fumigation are due largely to the conditions incidental to hibernation, viz., the reduced moisture content and comparative inactivity of the insects.

INTRODUCTION.

Nests of the brown-tail moth were discovered about January 4, 1909, upon imported seedlings in several of the local nurseries, and during succeeding weeks thousands of them were secured upon similar stock in this and other nursery centers of the State. Seedlings carrying nests were destroyed as soon as detected, but as the examination of the shipments progressed it was observed that many of the nests were broken, thus permitting the hibernating caterpillars to be scattered promiscuously among the trees. The question then arose as to the most effective means of disinfecting the shipments in addition to destruction of trees bearing winter nests. As the nurserymen were equipped with fumigation plants and were familiar with the use of hydrocyanic acid gas, fumigation was suggested. Preliminary tests by this process failed to kill the hibernating caterpillars at ordinary strengths and at the request of Dr. Raymond A. Pearson, then Commissioner of Agriculture, this Station undertook to determine the conditions which rendered fumigation ineffective. In this work it soon became apparent that temperature and humidity were important factors, and it is desired in this bulletin to call attention to the role played by these two factors under the peculiar conditions in which fumigation was attempted.

SOME REFERENCES TO MOISTURE AND TEMPERATURE IN FUMIGATION.

Lowe and Parrott[1] found in their fumigation experiments for the San José scale that the strength of gas uniformly effective during the spring, namely, .18 gram potassium cyanide per cubic foot, was wholly ineffective when the tests were made during the winter. Johnson[2] calls attention to some of the effects of temperature and moisture in fumigation. He especially mentions the increased injury to the plant when fumigations are made at high temperatures. He also states that, in the fumigation of nursery stock, the plants should be dry previous to treatment.[3] In speaking of the preparation of the greenhouse he says: "The room should not be fumigated immediately after the plants have been sprinkled or watered."[4] Townsend[5] has shown that the presence of moisture is very important in the fumigation of seeds. In his tests moist seeds absorbed the cyanide gas which injured the germinating qualities and rendered it unfit for food; while the dry seed was unaffected. Hinds and Turner[6] in a series of experiments with the rice weevil (*Calandra oryza* L.) found that carbon di-sulphide at the rate of 5 lbs. per 1000 cubic feet was effective when the work was done at a temperature above 70° F., while fumigation work done at temperatures ranging below 60° appeared to be largely ineffective. The moisture factor was taken into account in these experiments, but Hinds says "we have not found as yet that moisture has anywhere near the same importance as temperature".

In connection with the fumigation of citrus fruits in California, it has been generally assumed that if the work is carried on while the trees are damp injury to the foliage may follow. Penny[7] found in his studies that moisture on the leaves absorbed gas during the fumigation, though Gossard[8], Morrill[9] and Woglum[10] assert that the presence of moisture on the foliage is not important as affecting the results.

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[1] N. Y. Agr. Exp. Sta. Bul. 202, p. 206. 1901.
[2] Fumigation Methods, New York. 1902.
[3] L. c., p. 120.
[4] L. c., p. 128.
[5] Md. Agr. Exp. Sta. Bul. 75. 1901. L. c., p. 177.
[6] *Jour. Econ. Ent.* **3**:47. 1910.
[7] Del. Agr. Exp. Sta. 12th Rept., p. 212–237. 1900.
[8] Fla. Agr. Exp. Sta. Bul. 67, p. 648. 1903.
[9] U. S. Dept. Agr. Ent. Bul. 76:12–14. 1908.
[10] U. S. Dept. Agr. Ent. Bul. 90:68–69. 1911.

METHODS.

At the time the work was planned, no data were available regarding previous efforts to kill hibernating larvæ of the brown-tail moth by fumigation. There were, however, conflicting rumors regarding the success or failure of certain preliminary experiments conducted in this and other states. The writer inferred that most of the preliminary tests would be conducted in the laboratory while in many nursery establishments the fumigatorium is out-of-doors. To avoid confusion regarding the particular conditions under which the work was done a number of fumigators were arranged so that the work could be carried on at different temperatures. The experiments herein described include only those made with larvæ that had been separated from the nest. The method followed was to dissect the caterpillars out of the nest, and place them on a cheesecloth-covered wire frame. The container was placed near the center of the fumigatorium during the period of treatment. An arrangement to effect the combination of the chemicals after the closure of the fumigator was used in practically all tests. Following fumigation the condition of the larvæ was noted and the receptacle carrying the caterpillars placed under a bell jar, so that they might be kept under observation, other examinations being made during the next two or three days. If at any time subsequent to fumigation an individual showed signs of life it was counted as alive. The fumigators, which were designated by letters, are briefly described as follows:

A — Cubic contents 3.53 feet. A wooden box, made of tongued and grooved boards. Heavily coated on interior with hard paraffin.

B — Cubic contents 21.2 cubic feet. A wooden box lined with two thicknesses of glazed paper.

C — Cubic contents 31.5 cubic feet. Made of tongued and grooved material and lined with two thicknesses of building paper.

D — Cubic contents 17.6 cubic feet. Made of wood and coated on the interior with paint and shellac.

Bell jar.— Cubic contents 648.62 cubic inches. Made dark by means of a metal cylinder.

FUMIGATION EXPERIMENTS.

In addition to preliminary work using glass fruit jars, and some final checking up in which one of the large nursery fumigatoriums was employed, more than a hundred separate experiments with insects were made. Besides these, numerous experiments were carried out to determine the effects of similar charges of gas on the various kinds of seedlings. The experiments with caterpillars have been classified according to the amount of cyanide used per cubic foot in each fumigation. For the purpose of discussing the effects

of humidity and temperature, only the fumigations at .3 gram per cubic foot will be considered. The work as a whole is briefly summarized as follows:

Tests at .15 gram per cubic foot.—Thirty tests ranging in time from one to sixteen hours. Of the fumigations ranging from one to four hours none were effective, while some of the experiments of four to ten hours' duration were effective in that all of the caterpillars were killed. The tests of sixteen hours' duration were all effective.

Tests at .2 gram per cubic foot.—Five fumigations ranging in duration from three to fifteen hours gave results similar to those above.

Tests at .3 gram per cubic foot.—To be discussed fully later.

Tests at .6 gram per cubic foot.—Twenty-five fumigations ranging in duration from one to five hours. Of the fumigations lasting one hour none were effective, those of one and one-fourth partially, and those lasting four and five hours entirely effective.

Tests at .75 grams per cubic foot.—Three fumigations of one hour duration at temperatures of 33 to 70 degrees, all larvæ killed.

Tests at .9 gram per cubic foot.—Three fumigations of one hour duration ranging from 24 to 70 degrees F., the test at 24 degrees F. wholly ineffective.

TESTS WITH .3 GRAM OF CYANIDE PER CUBIC FOOT.

Description.— These consist of thirty-eight separate fumigations, that ranged in duration from one to sixteen hours. The number of individuals included in each test depended upon the number available at the time the test was made, at least ten larvæ being used in each experiment. The temperature was noted at the beginning and end of each fumigation and the average of these readings was taken as the temperature of the test. The condition of humidity was secured by the use of a sling psychrometer. The readings were taken frequently until the range of humidity surrounding each fumigator was learned, and from time to time during the course of the work.

Discussion.— By a study of the data in Table I, opposite, it will be noticed that the fumigations made at low temperatures are least effective as judged by the numbers surviving treatment. This difference is apparent by comparing individual fumigations or by a mass comparison of the data. It will be seen that, of twenty-seven fumigations ranging in time from one to four hours and at temperatures above 59 degrees F., ten were successful in that all the insects were killed. Opposed to this, six fumigations ranging in time from one to five hours at temperatures of 48 degrees or less were all unsuccessful. By combining the results of comparable experiments, we

TABLE I.— FUMIGATION OF LARVÆ AT .3 GRAM PER CUBIC FOOT.
Tests made during January, February and March, 1909, at Geneva, N. Y.

Number of test.	Fumigator used.	Location of fumigator.	Condition of Temperature and Humidity			Duration of test.	Total larvæ	Number of larvæ surviving.
			Dry bulb.	Depression of wet bulb.	Relative humidity.			
			Degs.	*Degs.*	*Per ct.*	*Hrs.*		
1	C	Barn	46	2	86	1	12	8
2	A	Museum	69½	13½	41½	1	10	7
3	B	Greenhouse	72½	3	86	1	10	1
4	A	Attic	48	.....	.....	1¼	23	21
5	A	Museum	52	(10)*	.....	1¼	14	1
6	A	Museum	59	(10)	.....	1¼	30	10
7	A	Museum	60	(10)	.....	1¼	15	6
8	A	Museum	65½	13½	39½	1¼	20	12
9	B	Greenhouse	67½	5	75½	1¼	15	3
10	B	Greenhouse	68	(6)	.....	1¼	16	0
11	B	Greenhouse	70	(6)	.....	1¼	17	2
12	B	Greenhouse	70	(6)	.....	1¼	13	0
13	B	Greenhouse	71	3	86	1¼	20	0
14	B	Greenhouse	75	(6)	.....	1¼	20	0
15	B	Greenhouse	75	(6)	.....	1¼	30	0
16	B	Greenhouse	80	.....	.....	1½	18	7
17	C	Barn	17	.....	.....	2	18	18
18	A	Museum	64	17	22	2	16	15
19	A	Museum	61	10	49	2	25	18
20	B	Greenhouse	76	10	59	2	30	20
21	A	Museum	60	10	48	3	10	6
22	B	Greenhouse	70	.....	.....	3	10	3
23	Bell jar	Laboratory	70	.....	.....	3	10	0
24	A	Laboratory	70	.....	.....	3	14	2
25	Bell jar	Laboratory	70	.....	.....	3	16	0
26	A	Museum	67½	6½	69	3	20	2
27	A	Laboratory	66	12	44	4	20	2
28	A	Laboratory	70	.....	.....	4	10	0
29	Bell jar	Laboratory	70	.....	.....	4	10	0
30	B	Greenhouse	68	2	90	4	23	1
31	Bell jar	Laboratory	70	.....	.....	4	20	0
32	A	Museum	70	.....	.....	4	20	5
33	C	Barn	34	.....	.....	5	10	3
34	A	Attic	48	.....	.....	5	10	9
35	Bell jar	Attic	48	.....	.....	5	19	2
36	A	Museum	72½	5½	75	6	20	0
37	A	Museum	67	4	80	10	20	0
38	B	Greenhouse	65	.....	.....	16	24	0

* Wet bulb reading not taken. The figures in parentheses are based on temperatures taken during other tests, (10) represents the minimum of the various wet bulb temperature in the museum and (6) the maximum of the wet bulb readings in the greenhouse. The conditions of humidity were determined by means of wet and dry bulb thermometers and a sling psychrometer.

secure the data in Table II, which are so chosen that the effects due to moisture are nil and the general percentages represent differences due mainly to temperature.

TABLE II.— COMPARISON OF FUMIGATIONS MADE AT TEMPERATURES HIGHER THAN 59° F. AND LOWER THAN 49° F.

Cyanide used at .3 gram per cubic foot. Data Selected from Table I.

SUMMARY OF SIX FUMIGATIONS MADE AT TEMPERATURES LESS THAN 49° F.				SUMMARY OF TWENTY-ONE FUMIGATIONS MADE AT TEMPERATURES ABOVE 59° F.			
Number of test.	Hours of fumigation.	Number of larvæ.	Number survived.	Number of test.	Hours of fumigation.	Number of larvæ	Number survived.
1.....	1	12	8	2–3...	1	20	8
4.....	1¼	23	21	7–15...	1¼	176	23
17.....	2	18	18	18–20...	2	71	53
33, 34, 35.	5	39	14	27–32...	4	103	8
Total.....		92	61	Total.....		307	92
Survived, 66 per ct.				Survived, 25 per ct.			

In Table II, it should be noted that in six fumigations ranging in time from one to five hours at temperatures less than 49° F., sixty-six per ct. of the caterpillars survived, while of twenty-one fumigations ranging in time from one to four hours at temperatures above 59° F., only twenty-five per ct. survived.

Even more striking are the effects of moisture, as will be seen by reference to Table III. These experiments, made under temperature conditions that were very similar, show that only three per ct. of the larvæ survived when the air was moist as opposed to fifty-six per ct. when the air was dry. In addition, five of the eight fumigations made under moist conditions were entirely successful in that all of the insects were killed, while none of the eight tests made in a dry atmosphere resulted in the death of all the caterpillars.

Of most interest in this connection is the combined influence of a humid atmosphere and a high temperature as compared with the opposite condition. The data at hand do not afford comparable experiments sufficient in number to make a comparison on this basis. But by an examination of the whole table the combined effect of temperature and moisture may be noticed. Of the twenty-five failures recorded in Table I, that is, experiments in which some of the larvæ survived the treatment, five were at a temperature lower than seventy, five were in a relative dry air, and six were made at both low

temperatures and in a dry atmosphere. Of the eleven successful fumigations recorded in Table I—i.e., all larvæ killed—seven were in a moist air at a temperature above 67° F. and the other four at a temperature of 70°.

TABLE III.— EFFECT OF MOISTURE ON THE PERCENTAGE OF CATERPILLARS KILLED.

Data taken from Table I.

EXPERIMENTS MADE IN AN ATMOSPHERE SHOWING A DIFFERENCE BETWEEN WET AND DRY BULB OF 10 TO 17 DEGREES.					EXPERIMENTS MADE IN AN ATMOSPHERE SHOWING A DIFFERENCE BETWEEN WET AND DRY BULB OF 3 TO 6½ DEGREES.				
Number of experiment.	Temperature of exposure.	Hours of fumigation.	Number of larvæ.	Number survived.	Number of experiment.	Temperature of exposure.	Hours of fumigation.	Number of larvæ.	Number survived.
	Degs. F.					*Degs. F.*			
2.....	69½	1	10	7	3.....	72½	1	10	1
5.....	52	1¼	14	1	9.....	67½	1¼	15	3
6.....	59	1¼	30	10	10.....	68	1¼	16	0
7.....	60	1¼	15	6	11.....	70	1¼	17	2
8.....	65½	1¼	20	12	12.....	70	1¼	13	0
18.....	64	2	16	15	13.....	71	1¼	20	0
19.....	61	2	25	18	14.....	75	1¼	20	0
20.....	70	2	30	20	15.....	75	1¼	30	0
	Av. temp. 63⅜ Survived, 56 per ct.		160	89		Av. temp. 64½ Survived, 3 per ct.		141	4

THE RESISTANCE OF THESE INSECTS TO FUMIGATION AND THE EFFECTS OF VARIATIONS OF TEMPERATURE AND MOISTURE ON THIS CONDITION.

The insects were in a state of hibernation, which is without doubt the cause of this peculiar resistance to the poison. By hibernation we understand that the insect is in a resting state and the water content of the body is reduced.[11] In the caterpillar the openings to the body are mouth, anus and spiracles. The spiracles are the exterior openings to the tracheæ, ramifying tubes in the body, that are filled with air and other gases. The air in the tracheæ is separated from the blood by the moist membrane of the tracheal wall. In respiration the air passes through the spiracles into the tracheæ from whence the gases diffuse through the moisture in the membrane into the fluids of the body cavity. The movement of the cyanide gas into the organs of respiration is similar to that of oxygen. In a dormant insect it enters the tracheæ largely by diffusion, while in an active specimen, the gas is drawn in by the movements of the seg-

[11] Tower, W. L. An Investigation of Evolution in Chrysomelid Beetles of the Genus *Leptinotarsa*. Carnegie Institution, Washington, D. C. No. 48, p. 245. 1906.

ments.[12] Hydrocyanic acid gas differs from oxygen in its strong affinity for water and greater solubility. Briefly, the amount of gas that an insect can take into its tissues in a given time depends upon the movement of gas through the spiracles and the condition of the tracheal wall with reference to moisture.

Temperature.— Changes in temperature very probably affect the hibernating animal in several ways, but of most importance in this connection is the rate of metabolism and the resulting reflex movements. With many species of animals and plants the effect of temperature changes has been observed.[13] It has been found that a rise in temperature is accompanied by an increase in the rate of respiration. It continues to increase as the temperature rises, until the injurious action of the high temperature becomes manifested by a decrease in the respiratory activity. This change in the rate of function of the protoplasm certainly has some bearing on the quickness of action of the gas but is probably not so important in affecting the action of the poison as are the reflex movements that result from the increase in temperature. The caterpillar has no breathing apparatus other than the bellows-like movement of the segments. As long as the caterpillar was quiet the gas could only enter by diffusion, but when the caterpillar begins to crawl there is a pumping action that greatly hastens the movement of the gas. The supply of caterpillars used in these experiments was kept at a temperature of thirty-five to forty-five degrees. While the insects were held at a low temperature they remained quiet, but when raised to seventy degrees or higher they were stimulated to crawl. It appeared in these experiments that much of the difference in the results of the work at different temperatures was due to the movements of the caterpillars.

Moisture.— Changes in the moisture content of plants and lower animals are correlated with changes in the rate of metabolism. This is very evident in seeds, and Kolkwitz[14] has shown that proportional amounts of carbon dioxide are excreted by seeds having different amounts of moisture. With certain low forms of animal life, such as rotifers, tardigrades and nematodes, continued loss of moisture produces a desiccation-rigor, during which metabolism proceeds at a very low rate.[15] As mentioned above, the phenomena of hibernation of insects, which is a condition of reduced activity, have been observed in some species to be accompanied by a relatively low moisture content. If an insect is placed in dry air it will lose moisture,[16] and we have determined that a fly pupa will absorb or lose moisture, depending upon its water content and the humidity of the surround-

[12] Sharp, David. Cambridge Nat. Hist. Vol. V, p. 130.

[13] Davenport, C. B. Experimental Morphology. Part I, p. 225. 1897.

[14] Jost. Plant Physiology, Ox. Ed. p. 341. 1907.

[15] Davenport, C. B. Experimental Morphology. Part I, p. 60. 1897.

[16] Bachmetjew, P. Experimental Entomologische Studien. Zweiter Band. pp. 53-61. 1907.

ing atmosphere. Thus the percentage of moisture may vary, which might alter the rate of metabolism, but this could not seriously affect the respiration of the caterpillars under discussion until they had been in the changed atmosphere long enough to absorb perceptible water, which would not occur in the interval of fumigation. The caterpillars used in these experiments were in a hibernating condition and hence probably low in body moisture. If the insect gained or lost moisture, a part of this interchange would take place through the walls of the tracheæ. This might be very important in altering the effect of the hydrocyanic acid gas, for the poison enters the system mainly by dissolving in the moisture of the tracheal wall. It is presumed that the tracheal wall of an insect with a low-water content would be in a dried condition as compared with that of a normal insect. Surround such an individual with dry air and the inner surface of the tracheal tubes would remain dry, a condition that would not favor a rapid solution of the gas. However, surround the same insect with a moist atmosphere and the dried wall would at once begin to absorb water at its outer surface, which would facilitate the formation of prussic acid. Thus a slight increase in the moisture of the air might add much to the speed and effectiveness of the gas.

Conclusion.— It appears from the above analysis that the unusual resistance of these caterpillars to fumigation is due to a condition of hibernation in which the moisture content of the body is low and the insects are comparatively inactive. It is possible that the spiracles are partially closed, which would also lessen the effect of the gas. A rise in temperature is correlated with greater effectiveness, but this increase in the deadly properties is more apparent between fifty and seventy degrees than at lower temperatures. A dry air is not favorable to fumigation with cyanide when the insects are in a hibernating state, which suggests that the low moisture content which usually accompanies this condition is one of the factors that help modify the effect of the gas.

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TECHNICAL BULLETIN No. 31. SEPTEMBER, 1913.

New York Agricultural Experiment Station.

GENEVA, N. Y.

THE ACTION OF RENNIN ON CASEIN.

ALFRED W. BOSWORTH.

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TECHNICAL BULLETIN No. 31.

THE ACTION OF RENNIN ON CASEIN*

ALFRED W. BOSWORTH.

SUMMARY.

A solution of calcium caseinate neutral to litmus and free from all other salts is not curdled by rennin.

A solution of calcium caseinate acid to litmus, which contains two equivalents of base for each molecule of casein, is curdled by rennin.

Solutions of ammonium, sodium or potassium caseinates are not curdled by rennin. In such solution, however, the casein is changed to paracasein, the paracaseinates of these bases being soluble.

When paracasein is produced from casein by the action of rennin no other substance is formed. Two molecules of paracasein are produced from each molecule of casein as a result of this action.

Rennin is not, strictly speaking, a coagulating ferment; the coagulation being a secondary effect, the result of a change in solubilities.

Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. It would follow from this that the action now attributed to rennin may be produced by any proteolytic enzyme. Work along this line is being carried out by the author.

In the light of the results reported in this paper together with those of Van Slyke and Bosworth the retarding action of soluble salts of ammonium, sodium and potassium on the coagulation of milk or casein solutions by rennin may be explained as follows: The addition of salts of these bases to milk or casein solutions results in a double decomposition whereby the calcium caseinate is changed to a caseinate of the base added. These are converted to paracaseinates by rennin, but owing to the fact that all the paracaseinates of these bases are soluble, no coagulation results.

INTRODUCTION.

The changes produced by the action of rennin in milk and solutions of casein have been the subject of many investigations. Fremy [1] was probably the first to give an explanation of this phenomenon. He believed the power to coagulate milk possessed by an extract of the mucous lining of a calf's stomach to be due to the presence

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[1] Fremy: *Ann. de pharm.* (Liebig), 31: 188, 1839.

of an enzyme which converts some of the lactose of the milk into lactic acid, the acid thus formed precipitating the casein.

Hammarsten [2] was the first to show that this coagulation of milk is due to the presence of a soluble ferment which acts directly upon the casein, producing, as he thought, two substances, the insoluble curd, *Käse*, which we call paracasein and a soluble product which he called whey-protein (*Molkeneiweiss*). He also showed that the change of casein to paracasein is independent of coagulation, the coagulation being due to the presence of soluble calcium salts.[3]

A great number of papers have been published upon this subject since the early work of Hammarsten.[4] As his explanation of the action of rennin has been generally accepted as correct, most of the recent investigations have been concerned with the influence of soluble salts upon the coagulation. These investigations have shown that the soluble salts of calcium, barium and strontium favor or hasten coagulation while salts of ammonium, sodium and potassium retard or inhibit coagulation.

Recently Van Slyke and Bosworth[5] have shown that casein and paracasein are acids having the same percentage composition; that the molecular weight of casein is probably 8888 ±, while the molecular weight of paracasein is one-half that of casein; that both have a combining equivalent of 1111; that combinations of casein or paracasein with one equivalent of calcium, barium or strontium are insoluble in water while the combinations with one equivalent of ammonium, sodium or potassium are soluble; and that ammonium, sodium or potassium caseinates can be changed by rennin to paracaseinates which are soluble and are precipitated by calcium chloride as calcium paracaseinates.

These facts would seem to indicate three things:

First, that rennin action consists of the hydrolytic splitting of the casein molecule into two similar molecules of paracasein; perhaps in somewhat the same manner that maltose is split into two molecules of dextrose.

Second, that, as a consequence of this cleavage it would seem to be doubtful if Hammarsten's whey-protein could be one of the products of rennin action.

Third, that rennin is not, strictly speaking, a coagulating ferment, the coagulation of paracasein being due to the fact that calcium paracaseinates are less soluble than the calcium caseinates, especially in the presence of soluble salts of calcium, barium or strontium.

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[2] Hammarsten: *Maly's Jahresbericht*, 1872, p. 118; 1874, p. 135; 1877, p. 158.

[3] See also Arthus and Page: *Arch. de physiol.* (5th series), ii.

[4] An excellent review of the literature with references may be found in Bulletin 56 of the Hygienic Laboratory of the Public Health and Marine Hospital Service of the United States.

[5] Van Slyke and Bosworth: *Jour. Biol. Chem.*, 14:203–236.

EXPERIMENTAL

This investigation was undertaken as an attempt to determine the truth of the statements made above. In repeating the work of Hammarsten and others a soluble substance which had not been coagulated by rennin and could not be precipitated by dilute acetic acid was always found in the filtrate. Casein solutions for such investigations have been prepared, as a general rule, by shaking pure casein with an excess of lime water or by grinding with moist calcium carbonate. The casein solutions thus obtained were made neutral to litmus and coagulated by the addition of rennin. The curds were filtered off and the filtrates examined for nitrogen. Soluble nitrogen was always found, but the amounts were not constant and seemed to have no relation to the amounts of casein or rennin used. In the control experiments, to which no rennin had been added, similar amounts of nitrogen which could not be precipitated by dilute acetic acid were also found.

Caseinate solutions prepared in the manner described contain basic caseinates, either neutral or alkaline to phenolphthalein. As Robertson [6] has shown that such caseinates in solution undergo an autohydrolysis, the following experiment was carried out in order to determine if this might account for the soluble nitrogen found.

Five grams of casein were dissolved in 250 cc. of $\frac{N}{50}$ calcium hydroxide in the presence of toluol. After complete solution of the casein, portions of the solution were withdrawn at intervals and the casein precipitated by means of dilute acetic acid. The casein was filtered off and the nitrogen in the filtrates determined by the microchemical method devised by Folin.[7] The results are as follows:

	3 hours	15 hours	24 hours
Milligrams of nitrogen in original solution	158	158	158
Milligrams of nitrogen not precipitated by rennin	4.0	10.0	28.8

Results of the same nature were obtained with solutions made by grinding casein with moist calcium carbonate. The extent of this autohydrolysis, temperature being constant, depends upon time. As dry casein goes into solution very slowly and freshly precipitated casein is quite rapidly redissolved the following procedure was adopted in order to circumvent autohydrolysis.

Ten grams of pure dry casein were dissolved in 500 cc. of $\frac{N}{50}$ calcium hydroxide. The casein was then precipitated by adding about

[6] Robertson: *Jour. Biol. Chem.* 2:344; see also Osborne: *Jour. Physiol.*, 27:398.

[7] Folin and Farmer: *Jour. Biol. Chem.*, 11: 493. All nitrogen determinations reported in this paper were made by this method.

250 cc. of $\frac{N}{25}$ acetic acid, the liquid was siphoned off, the casein washed several times with water, placed in a linen bag and squeezed as dry as possible. It was then transferred to a mortar, ground to a paste with a little water, the paste put into a flask and 150 cc. of water, 75 cc. of lime water and some toluol were added to it. After considerable shaking the lime water became saturated with casein. By this process a solution was obtained containing a calcium caseinate neutral to litmus but acid to phenolphthalein,[8] and containing four equivalents of base. The undissolved casein was removed by centrifuging and filtering. The amount of casein in solution was determined and the solution so diluted that each 50 cc. contained 1 gram of casein. Fifty cc. portions of this solution were withdrawn at intervals and precipitated with acetic acid. The amounts of nitrogen found in the filtrates were as follows:

	30 minutes	5 hours	12 hours	24 hours
Milligrams of nitrogen in original solution	158	158	158	158
Milligrams of nitrogen not precipitated by acetic acid	0.07	0.92	1.96	2.00

Casein solutions prepared in this manner gave the following reactions. *They were not coagulated by rennin.* The addition of a few drops of a 10 per ct. solution of calcium chloride caused them to curdle;[9] the addition of one drop caused no change but the subsequent addition of rennin produced coagulation. If enough hydrochloric acid was added to change the caseinate to one containing two equivalents of calcium,[10] the addition of rennin caused coagulation. That this coagulation was not due to the calcium chloride formed by the acid was shown by the fact that rennin caused coagulation after all this calcium chloride had been removed by dialysis. In both instances *the coagulation removed all the nitrogen from the solution,* as is shown by the following figures:

Milligrams nitrogen in original solution	Milligrams nitrogen not precipitated by rennin
316	0.8
316	0.6
316	0.2±

The behavior of such caseinate solutions toward rennin can be explained by the work of Van Slyke and Bosworth as follows:

[8] Robertson: *Jour. Biol. Chem.*, 2: p. 317; Van Slyke and Bosworth: *ibid.*, 14: p. 211–225.

[9] Robertson: *ibid.*, 2: 381. Robertson believes that the addition of the common Ca ion represses the dissociation of the caseinate and thus causes precipitation.

[10] Van Slyke and Bosworth: *ibid.*, 14: pp. 211–225.

A molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in pure water but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate each containing one equivalent of base. Such a paracaseinate is insoluble in pure water.

The small amounts of nitrogen recovered in the filtrates in the experiments given above may be due to autohydrolysis or to proteolysis produced by the pepsin in the rennin extract used, as is indicated by the following experiment.

Into each of several flasks were placed 50 cc. of a casein solution and a little toluol. One-half of the flasks received a few drops each of rennin solution, the others being kept as controls. The contents of the flasks were examined at intervals for autohydrolysis and proteolysis. The nitrogen in the control flasks which was not precipitated by acetic acid was considered as due to autohydrolysis; while in the case of the other flasks the nitrogen not removed by filtering was considered to be due to autohydrolysis and proteolysis. By subtracting the nitrogen found in the controls from those containing rennin a fair idea as to the extent of the proteolysis might be obtained.

	30 minutes	12 hours
Milligrams of nitrogen in original solution as casein	158	158
Milligrams of nitrogen in filtrate from rennin flasks	3.4	18.2
Milligrams of nitrogen in filtrates from control autohydrolysis	0.1	2.1
Milligrams of nitrogen due to proteolysis	3.3	16.1

Solutions of ammonium, sodium or potassium caseinates containing two or more equivalents of base could not be coagulated by rennin, but the subsequent addition of calcium chloride caused coagulation, the curd being calcium paracaseinate. That sodium caseinate in solution was changed to sodium paracaseinate was shown by the following experiment. Rennin was added to a solution of sodium caseinate and after a short time acetic acid was added. The precipitate, after being purified and dried, was found to be paracasein.

In conclusion I wish to express my appreciation of the interest in this work shown by Dr. L. L. Van Slyke, of the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y., and Dr. Otto Folin of the Biochemical Laboratory of the Harvard Medical School, Boston, Mass.

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